acta physiologica latino americana

VOLUMEN VIII
Nº 4

1958

ACTA PHYSIOLOGICA LATINOAMERICANA

Editada por la Asociación Ciencia e Investigación

Esta revista se edita con la ayuda del Fondo Alejandro Menéndez Behety

SECRETARIO DE REDACCION
Dr. Manuel R. Malinow
Avda. R. Sáenz Peña 555, Buenos Aires — Argentina

ADMINISTRADORA
Srta. Josefina Yanguas

Suscripción

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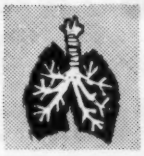

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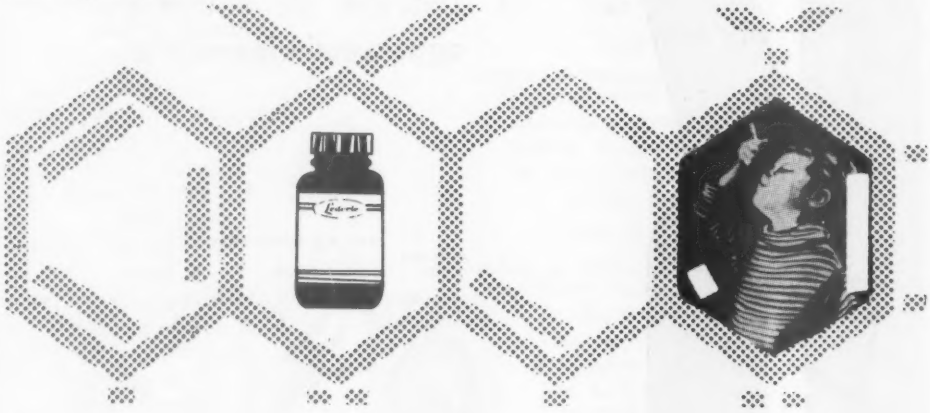

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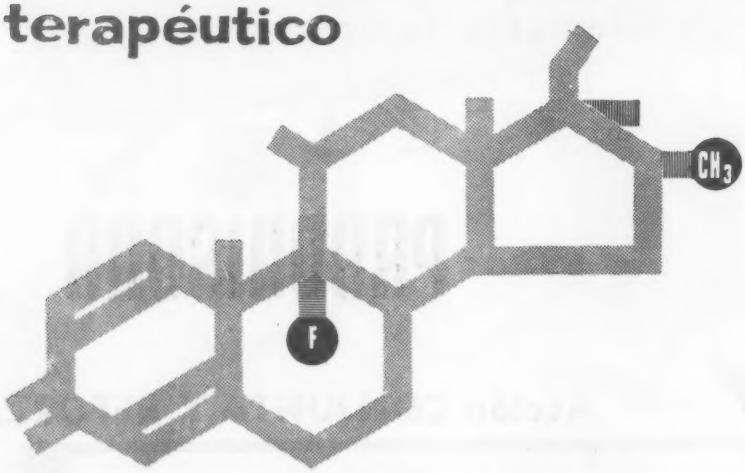

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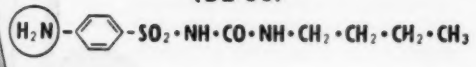

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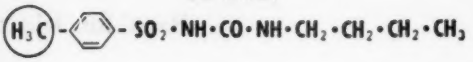

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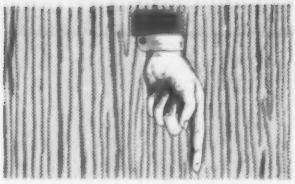

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ELEAZAR S. GUZMAN BARRON

(1899 - 1957)

"Tengo el anhelo de que las Repúblicas Latinoamericanas sigan definitivamente por el camino de la investigación médica." Esta frase pronunciada por Eleazar Guzmán Barrón en una conferencia que dió en Montevideo ("Ciencia

e Investigación", 1957, *13*, 150) sintetiza uno de sus más queridos deseos. Sabía que la tarea era formidable, pero conocía bien la situación y los puntos hacia donde debían concentrarse los esfuerzos.

Es interesante reproducir también otras partes de la mencionada conferencia, puesto que ellas dan una idea de la manera de pensar de su autor y porque, además, representan la opinión sincera y franca de un hombre imparcial empeñado en una crítica constructiva. Así dijo también con toda franqueza: "Tengo que decir y lo digo con dolor que no hay Universidades en Latinoamérica, y no hay universidades en Latinoamérica porque, ni se tiene el concepto esencial, de que la Universidad es investigación y es enseñanza, ni se sigue el concepto esencial de que la universidad representa dedicación exclusiva."

Comentó también algunos aspectos de la organización universitaria en los siguientes términos: "Alguien en Perú me preguntaba qué pensaba yo del co-gobierno y mi respuesta fué, que en el momento en que las Universidades se convirtieran en Universidades, el momento en que el maestro viviera en el claustro, en contacto continuo con el estudiante, el co-gobierno desaparecería. Yo creo que el co-gobierno es la consecuencia de que el alumno ve al Profesor sólo al frente de un pupitre; el Profesor es algo intangible."

También le preocupaba la práctica tan difundida de admitir, sin discriminación, un elevado número de estudiantes. Así dijo: "El segundo postulado que requiere la creación de una escuela médica moderna es la limitación del número de estudiantes." "Una escuela médica moderna es aquella que hace enseñanza experimental e individual", "me atrevería a decir que una Facultad de Medicina que recibe más estudiantes, que lo que ella está capacitada para dar enseñanza experimental e individual, hace daño a la Nación y hace daño al estudiante".

Estas son las ideas generales que expuso Guzmán Barrón en su conferencia, las presentó y discutió en detalle para luego explicar los planes de estudio concebidos para la nueva Facultad de Medicina de Arequipa, Perú. Había participado activamente en la organización de esta escuela. En ella tenía puestas todas sus esperanzas. Quería ver crecer una Universidad totalmente distinta de las tradicionales de Latinoamérica. Deseaba que en ella se dedicara todo el tiempo a aprender, enseñar e investigar. Como se ve, los lazos que unían a Guzmán Barrón con Latinoamérica no se debilitaron aunque estuvo unos treinta años fuera de su patria. No sólo no se debilitaron los lazos, sino que parecía que cada vez tiraban más.

Había nacido en Huari, Ancash, Perú. Cursó estudios primarios en esa misma ciudad en un colegio, cuyo director era su padre, Don Sebastián V. Guzmán. Recibió su título de Doctor en Medicina en la Universidad de San Marcos, de Lima, en 1924, y, dado que obtuvo la mejor nota de su promoción, el Gobierno Peruano le otorgó una beca para perfeccionamiento en el extranjero. Pudo satisfacer así, sus deseos de profundizar más sus conocimientos médicos. Pasó dos años en Francia con el Prof. Chauffard en la Clínica de St. Antoine, en París, y con el Prof. Blum, en la Clínica de Estrasburgo. En 1927 llegó a los Estados Unidos, becado por la Fundación Rockefeller, y fué a trabajar con Leonor Michaelis en la escuela de Medicina de Johns Hopkins en Baltimore. Esto debió representar un gran cambio: de la clínica a la Físicoquímica aplicada a la Biología.

Con Michaelis trabajó sobre grupos sulfhidrilo y el interés que se le despertó sobre este tema lo conservó durante toda su carrera. Fué en esta época, que descubrió que el agregado de azul de metileno a los glóbulos rojos determina

un aumento en el consumo de oxígeno. Esta observación al parecer pequeña, tuvo un importante papel en el desarrollo de la bioquímica. Sirvió de punto de partida para Otto Warburg en sus estudios de oxidación del glucosa-6-fosfato, que culminaron en el descubrimiento del trifosfopiridinnucleótido, y de las flavoproteínas.

En 1930 pasó al Departamento de Medicina de la Universidad de Chicago. Allí dirigía una sección de Bioquímica, como profesor asistente. En 1942, fué nombrado profesor asociado, en 1952 profesor. Aquel laboratorio fué uno de los más activos, gran cantidad de graduados pasaron por allí y se iniciaron en la investigación bioquímica. El laboratorio de Guzmán Barrón era, además, un punto de reunión de los latinoamericanos. Allí, Achito, como le llamaban en confianza, los recibía, los estimulaba y les exponía sus planes para crear una verdadera Universidad, en algún sitio de Latinoamérica. Toda iniciativa en este sentido era bien recibida y hubo muchas que fracasaron porque América Latina es tierra fértil para improvisaciones y para éxitos fáciles, pero árida para lo que represente trabajo paciente y serio.

Los estudios de Guzmán Barrón se relacionaron con aspectos fundamentales del metabolismo celular; oxidación de ácido láctico en bacterias, determinación del potencial de óxido reducción del sistema lactato-piruvato y de los hemocromógenos, y varios aspectos de la utilización del ácido pirúvico. Durante la última guerra mundial, tomó parte en algunas investigaciones aplicadas. Fué entonces que se interesó en los gases tóxicos y en el mecanismo de acción de las radiaciones. Este nuevo problema lo relacionó con el trabajo iniciado con Michaelis en Johns Hopkins y es así que publicó trabajos sobre influencia de las radiaciones sobre los grupos —SH.

Durante muchos años Guzmán Barrón aprovechó las vacaciones para ir a la estación marítima de Woods Hole. Allí alternaba el descanso con el trabajo y ampliaba sus conocimientos de Biología. En los últimos años reemplazó las vacaciones de Woods Hole por visitas a Sudamérica y en particular al Brasil. Allí enseñaba, aconsejaba y trataba de despertar vocaciones. Su influencia ha sido grande. A sus méritos de inspirado y preciso investigador hay que sumar los de impulsador y consejero de la Bioquímica Latinoamericana. Es de esperar que los amigos, y los que estuvieron bajo su influencia y aprendieron con él, vean algún día la materialización del sueño y de los anhelos de Eleazar S. Guzmán Barrón.

L. F. L.

THE ENZYMES FOR RIBOSE-5-PHOSPHATE METABOLISM IN BLOOD SERUM OF NORMAL INDIVIDUALS AND CANCER PATIENTS (*)

E. S. Guzmán Barrón †, A. Cazorla, M. I. Mello, and A. B. Hargreaves

(Department of Medicine, Chemistry Division, the University of Chicago and Instituto de Biofisica, Facultade de Medicina, Universidade do Brasil, Rio de Janeiro).

IN MEMORIAM

The present paper is the result of Profesor E. S. Guzmán Barron's research work in the middle of 1955, that was initiated at the Departamento de Biofisica, Universidade do Brasil, Rio de Janeiro, in collaboration with Drs. M. I. Mello and A. Hargreaves. Upon his return to the University of Chicago the investigation was continued in collaboration with Dr. A. Cazorla and the manuscript drafted.

It is in honor of the memory of such a thorough investigator endowed with exceptional human qualities, that these unpublished findings are presented in Acta Physiologica Latino Americana.

It is gratifying that Profesor Guzmán Barrón's original findings have been confirmed by F. H. Bruns in his paper —Nachweis und Aktivitatsmessung von 5-Phosphoriboisomerase in Blutserum— published in Biochemische Zeitschrift, 327, 523 (1956).

RIBOSE-5-PHOSPHATE appears to be metabolized in cancer cells at greater rates than in normal cells. Villavicencio and Guzman Barron reported this observation (1, 2) and it has been confirmed by Kit (3) and Racker (4). Ribose-5-phosphate is an intermediate in the conversion of monosaccharides to pyruvic acid when this conversion occurs through the hexose monophosphate shunt. Three enzymes were chosen for our study: Phosphoriboisomerase, transketolase and transaldolase. All three participate in reactions of the hexose monophosphate shunt.

We have considered the possibility that these enzymes exist in serum of normal subjects and that their levels are higher than in the serum of cancer patients. Many enzymes appear in the serum and their levels often reflect tissue levels. We have examined the serum from several normal subjects, some

(*) Aided by the Office of Naval Research, Department of Navy, U. S. A.

(†) Deceased.

Part of this work was carried out at the University of Chicago by one of us (A. C.) under the auspices of a Guggenheim Fellowship.

cancer patients and pregnant women. Differences in enzymatic activity were observed. These differences are significant and they are reported in this paper.

Materials and methods

All determinations were made in serum from fasted subjects. The preparation of serum was carried out at 4° C. Determinations were made in 1 ml of serum. Substrate, buffers and other additions were made and the mixture incubated at 37° C. In one of the series (Table I) the incubation period was 120 minutes. In all others, incubations were carried out for 60 minutes. Control measurements were made using identical conditions but enzymatic activity was destroyed by adding trichloroacetic acid to the serum before adding the substrate.

Pentose was determined by the method of Mejbaum (5). Heptulose by the same method. Values for heptulose were computed using the calculations suggested by Horecker and collaborators (6). Fructose was determined by the method of Roe (7).

Procedure

First series (Table I). 1 ml of serum was incubated at 37° C for 120

TABLE I

Utilization of ribose-5-phosphate. Incubation period: 120 minutes

Normal serum		Cancer patients	
Nº	μM/100 ml	Diagnosis	μM/100 ml
1	335	Adenocarcinoma, stomach	392
2	224	Scirrhous carcinoma, stomach	565
3	150	Adenocarcinoma, stomach	405
4	350	Carcinoma, prostate	487
5	260	Carcinoma, mammary	915
		Carcinoma, epidermoid, stomach ..	672
Average ± S. E.	262 ± 36		573 ± 74

Normal vs. cancer serum, $p < 0.01$.

minutes, with 1 ml of a 0.01M solution of ribose-5-phosphate in the sodium salt form. At the end of the incubation period, 2 ml of a 10 % trichloroacetic acid solution were added. The precipitated proteins were removed by centrifugation. The supernatant was diluted and used for the determinations just described.

Second series (Tables II and III). 1 ml of serum was incubated at 37° C for 60 minutes with 0.5 ml of a 0.02M solution of ribose-5-phosphate and 1 ml

TABLE II

Normal serum

Nº	Utilization of ribose-5-phosphate	Formation of heptulose	Formation of fructose
1	134	6.2	7.5
2	98	0.0	12.0
3	149	12.5	2.5
4	116	18.1	8.5
5	119	13.1	6.0
6	141	17.5	9.5
7	114	12.5	10.0
8	166	22.5	14.5
9	133	17.5	14.5
10	117	14.4	10.0
11	79	8.1	7.0
Average	± S. E. 124 ± 7.2	14.2 ± 1.5	9.3 ± 1.0

All values are given in micromoles, per 100 ml of serum.
Incubation period: 60 minutes.

of a 1M Tris buffer pH 7.4 At the end of this period, 2.5 ml of a 10 % trichloroacetic acid solution were added. The precipitated proteins were removed by centrifugation and the supernatant fluid used for the determinations described.

TABLE III

Serum from cancer patients and pregnant women

Diagnosis	Utilization of ribose-5-p	Formation of heptulose	Formation of fructose
Melanoma, liver metastasis	264	84.4	54.0
Adenocarcinoma, ovary	225	38.7	37.0
Carcinoma, mammary	196	43.8	16.0
Melanoma, brain metastasis	217	33.8	13.5
Carcinoma, mammary, bone metastasis	203	35.0	12.0
Carcinoma, mammary, bone metastasis	177	30.6	16.0
Average ± S. E.	214 ± 10.3	42.4 ± 7.2	23.5 ± 5.9
Pregnancy	215	24.0	4.4
Pregnancy	227	47.7	6.7
Pregnancy	204	—	—
Pregnancy	184	12.0	6.9
Pregnancy	163	2.4	8.3
Average ± S. E.	198.6 ± 13.4	21.5 ± 9.7	6.6 ± 0.8

All values are given in micromoles per 100 ml of serum.
Incubation period: 60 minutes.

Results

In Table I are reported values (in micromoles per 100 ml of serum) for the utilization of ribose-5-phosphate in serum from normal subjects and cancer patients. The incubation period in these series was 120 minutes. Values obtained for serum from normal subjects are significantly lower than the values obtained for serum from cancer patients. The average for normal serum (5 subjects) was 262 micromoles, and the average for serum for six cancer patients was 527 micromoles. The difference is significant ($p < 0.01$).

In Table II, are given values for the disappearance of ribose-5-phosphate and formation of heptulose and fructose in 11 normal sera. In Table III are given values for serum from cancer patients and also serum from pregnant women. A comparison of these tables reveals that there are significant differences in all instances. In normal serum the disappearance of ribose-5-phosphate proceeds at a rate of 124 micromoles per hour, whereas in serum from cancer patients it disappears at a rate of 214 micromoles per hour. The difference is significant ($p < 0.001$). In serum from pregnant women the rate of disappearance is also higher than in normal serum but not as high as in serum from cancer patients. The formation of heptulose in serum from cancer patients proceeded at a much higher rate than in serum from normal subjects, namely at 14.2 micromoles per hour in normal and 42.4 micromoles per hour in cancer. The difference again is significant ($p < 0.01$). Finally, the formation of fructose followed the same trend, i. e., it was higher in serum from cancer patients than in serum from normal subjects. The difference is also significant.

Discussion

The utilization of ribose-5-phosphate and the formation of heptulose and fructose demonstrate that in human serum are present enzymes that participate in the hexose monophosphate shunt, viz; phosphoriboisomerase, transketolase and transaldolase. In all instances we have demonstrated a significant difference in the concentration of these enzymes when serum from normal subjects is compared with serum from cancer patients. In the latter, ribose-5-phosphate disappears at a greater rate and heptulose and fructose are formed at greater rates. The presence of these enzymes in serum can be explained in a number of ways. For example, it is possible that a continuous cytolysis of white blood cells allows the passage of these enzymes from the tissues across the lymphatic barrier. In cancer patients this effect is probably more pronounced inasmuch as cancer cells contain more of these enzymes than normal cells do [1, 2]. In the case of pregnant women, the increase of these enzymes could be ascribed to a greater production of them by the human embryo. Studies made by us have shown that, at least in the chick, the utilization of ribose-5-phosphate proceeds at a greater rate in the embryo liver than in adult tissues [8].

We have been unable to demonstrate pentolysis of ribose. This is in agreement with observations made by others [9, 10]. Pentolysis was reported by Menckes [11, 12].

Summary

Enzymes participating in the metabolism of ribose-5-phosphate, viz: phosphoriboisomerase, transaldolase and transketolase were measured in serum.

They were determined by the disappearance of ribose-5-phosphate and formation of heptulose and fructose. Serum from cancer patients and pregnant women utilize ribose-5-phosphate at a greater rate than serum from normal subjects. Similarly, the formation of heptulose and fructose was greater in cancer patients. We found no pentolysis of ribose.

References

(1) Villavicencio, M., Guzmán Barrón, E. S.: *Fed. Proc.*, 1955, *14*, 297.
(2) Villavicencio, M., Guzmán Barrón, E. S.: *Arch. Biochem. Biophys.*, 1957, *67*, 121.
(3) Kit, S.: *Cancer Res.*, 1956, *16*, 70.
(4) Racker, E.: *Ann. N. Y. Acad. Sci.*, 1956, *63*, 1017.
(5) Mejbaum, W. Z.: *Z. physiol. Chem.*, 1939, *258*, 117.
(6) Horecker, B. L., Smyrniotis, P. Z., Klenow, H.: *J. biol. Chem.*, 1953, *205*, 661.
(7) Roe, J. H.: *J. biol. Chem.*, 1934, *107*, 15.
(8) Cazorla, A., Guzmán Barrón, E. S.: *Exp. Cell. Res.*, 1958, *14*, 68.
(9) Steen, S. N.: *J. Nat. Cancer Inst.*, 1951, *12*, 195.
(10) Roe, J. H., Cassidy, J. W., Tatum, A. C., Rice, E. W.: *Cancer Res.*, 1952, *12*, 238.
(11) Menckes, G.: *Helv. med. acta*, 1951, *18*, 125.
(12) Menckes, G.: *Gastroenterology*, 1952, *78*, 192.

PATHWAYS OF GLUCOSE METABOLISM IN RABBIT CEREBRAL CORTEX (*)

MARINO VILLAVICENCIO, FERMÍN ROSALES, ARTURO OLIVERA, ERNESTO MELGAR, AND ROSA GUERRA

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THE IMPORTANT routes of carbohydrate metabolism that are known to occur in mammalian cells are: a) the Embden-Meyerhof glycolytic pathway; b) the phosphorylated oxidation, the so called Warburg-Dickens pathway, hexose monophosphate shunt, pentose phosphate pathway; and c) the uronic acid pathway which has come to light recently (1). The existence of these different pathways and the extent to which each is followed can be studied by the detection and measurement of the relative activities of the different enzymes that take part in them, by separation of these pathways by specific inhibitors, by detection of key intermediates, and finally, labeled substrates may be used to obtain information on the route through which the different intermediates have passed. Use of only one of these methods may give erroneous answers (2), as shown in the contradictory conclusions reached by many investigators who used the single approach. For example, Barker et al. (3) in a study of the metabolism of glucose in brain slices, using iodoacetate as an inhibitor of glycolysis, concluded that it proceeded via phosphorylative oxidation, whereas Fuhrman and Field (4) with the same technique concluded that glycolysis was the main pathway. In the studies on the aerobic metabolism of glucose in rabbit cerebral cortex presented in this paper, we have used all the techniques mentioned above in order to reach a closer approximation to what happens in the cell, and we have obtained evidence that the Embden-Meyerhof glycolytic pathway operates almost exclusively in this tissue.

EXPERIMENTAL

The Preparation of Slices and Cell-free Extracts from Rabbit Cerebral Cortex

For the preparation of slices of rabbit cerebral cortex, normal adult rabbits were killed by decapitation, the brain removed and put into a beaker

(*) This work was supported by grants from the Rockefeller Foundation, the Kellogg Foundation and in part from the Faculty of Medicine, University of San Marcos.

containing ice-cold isotonic KCl. Thin sections of cerebral cortex parallel to the outer surface were cut with a razor-blade (5). The slices were kept below 3° C until they were weighed and introduced into Warburg vessels. For studies on aerobic glycolysis, vessels contained Krebs-Ringer phosphate (6) with a high potassium content (40 parts of 0.154 M. KCl and 64 parts of 0.154 M. NaCl. The proportion of the other ingredients were not changed) to stimulate glucose metabolism (7). The pH of the buffer was 7.4. The incubations were carried out at 38° C with the usual Warburg manometric technique. At the end of the experiments (usually 2 hrs.) proteins were precipitated with $ZnSO_4$ and Ba $(OH)_2$. The supernatant fluid was obtained after centrifugation and kept for chemical analysis.

Cell-free extracts were prepared by dispersing rabbit cerebral cortex in 9 vol. of ice-cold 0.154 M. KCl in a Potter-Elvehjem hand homogenizer. All subsequent operations were carried out below 3° C. The cell debris was removed first in the International refrigerated centrifuge, model PR-2, at 600 x g. The supernatant fluid was further centrifuged in the multispeed attachement for 30 minutes at 24 000 x g. The fluid was then dialyzed overnight against 0.154 M. KCl at 3° C. in a cellophane bag.

Substrates and Coenzymes

The barium salts of glucose-6-phosphate, fructose 1,6-diphosphate and ribose-5-phosphate were obtained from Schwarz and Co.; di-and triphosphopyridine nucleotide from Pabst and Co.; glucose oxidase and the barium salt of 6-phosphogluconic acid from Sigma Chemical Co.; iodoacetic acid from Eastman Chemical Co. was recrystalized with carbon tetrachloride. Glucose-1-C^{14} and glucose-U-C^{14} (*) from Nuclear Instruments and Chemical Co.; Glucose-6-C^{14} from Research Specialties, California; glucose-2-C^{14} from Dr. E. S. G. Barron.

Methods

Lactic acid was determined by the method of Barker and Summerson (8); glucose, enzymatically with glucose oxidase (9); total nitrogen of the cell-free extracts, Ma and Zuazaga (10); ribose and sedoheptulose, Mejbaum (11); For the last determination, the equation of Horecker et al. (12) was adapted to our experimental conditions; fructose, Roe (13). Organic acids were extracted with ether and separated in the silica gel column according to Bulen et al. (14).

The experiments with labeled substrates were performed in large Warburg vessels with two side arms in a total volume of 11.5 ml. Substrate was added from one side arm, and, at the end of the incubation period, 0.5 ml. of 5 N H_2SO_4 was added from the second side arm to stop the reaction and to liberate CO_2 from the buffer. The released CO_2 was collected by adding 0.5 ml. of 20 % NaOH through the ventling plug and shaking the flask until the CO_2 was absorbed. In this way the absorbed CO_2 as well as the evolved CO_2 could be determined. With a pipette bent at the tip, $Na_2C^{14}O_3$ was transferred to a sodium bicarbonate apparatus (15) containing 400 μM of sodium carbonate as diluent; the CO_2 was released by adding 5 N H_2SO_4 and then absorbed in a Ba $(OH)_2$, $BaCl_2$ solution (16) to transform the CO_2 into $BaCO_3$. Protein precipitation was carried out according to Neish (17). Lactic acid and glucose

(*) Uniformly labeled glucose.

were determined on an aliquot of the supernatant fluid. The remaining portion was acidified with H_2SO_4 and extracted with ether for 18 hrs. The organic acids from the ether extract were separated in a silica gel column. Radioactive glucose and purified lactic acid were transformed into $BaCO_3$ by the wet combustion method of Van Slyke and Folch (18), and degradation of the different carbons of lactic acid, according to Sakami et al. (15). Radioactivity measurements of $BaC^{14}O_3$ were made in a model 192 Ultrascaler with D-47 Gas Flow Counter (Nuclear Chicago). The counts per minute were corrected for self absorption by extrapolation to infinite thinness.

The activity of the enzyme systems in the cell-free extracts was determined according to Ghiretti and Barron (19).

Results and discussion

Enzymes for Glycolysis and Pentose Phosphate Pathway in Cell-free Extracts

It is well established that an essential condition for the occurence of a metabolic pathway in cells and tissues is the presence of enzymes required for that pathway, but the presence of an anzyme in a cell-free extract does not necessarily mean that this enzyme is actually used by the cell. For example, glucose dehydrogenase is present in liver, although glucose metabolism in this tissue does not proceed via direct oxidation. Cell-free extracts of rabbit cerebral cortex contain enzymes for glycolysis and pentose phosphate pathway and phosphoglyceraldehyde dehydrogenase is more active than any one of the enzymes of pentose phosphate pathway (Table I). We have also detected fructose formation from ribose-5-phosphate, which means that all the enzymes for pentose phosphate shunt are present in rabbit cerebral cortex. This tissue thus possesses the capacity to utilize glucose through the Embden-Meyerhof glycolytic process and the pentose phosphate shunt. A number of investigators (20, 21, 22, 23) have also reported that brain contains enzymes for the metabolism of glucose through both pathways.

TABLE I

The activity of enzymes for glucose metabolism in dialyzed cell-free extracts from rabbit cerebral cortex

One unit = 1 mμM of substrate or coenzyme reacting/min./mg. protein at 24° ± 1° C.

Enzyme	Units
Phosphoglyceraldehyde dehydrogenase	29.0
Lactic dehydrogenase	7.8
Glucose 6-phosphate dehydrogenase	5.6
Phosphogluconic acid dehydrogenase	7.8
Ribose-5-phosphate utilization	12.5
Sedoheptulose formation	5.1

Effect of Iodoacetate on Respiration and Aerobic Glycolysis

Lundsgaard (24) discovered that anaerobic glycolysis could be prevented by iodoacetic acid and showed that proper concentrations of this acid would inhibit fermentation by yeast without affecting respiration. From these experiments,

Lundsgaard concluded that oxidation might be independent from the products of anaerobic breakdown. Similar claims have been made by Barker et al. (3) for animal tissues. Fuhrman and Field (4) found that fermentation and respiration in mammalian brain are not separable by treatment with iodoacetate. No conclusion could be derived from these experiments regarding the pathways of glucose metabolism because simultaneous measurements were not made of glucose utilization and lactic acid or alcohol formation, and because it was not considered that anaerobic glycolysis is more affected by iodoacetate than aerobic glycolysis, since phosphorylation of glucose is another limiting factor in the absence of oxygen (2). Therefore, if respiration is not affected by iodoacetic acid concentrations able to inhibit anaerobic glycolysis, it cannot be taken as proof that respiration is chemically independent from anaerobic breakdown.

TABLE II

Effect of iodoacetate on the respiration and aerobic glycolysis of rabbit cerebral cortex slices

Cerebral cortex slices were incubated with 30μM of glucose in Ringer-phosphate as indicated in Methods. Temp. 38° C.; O_2 as gas phase. Total volume, 3.15 ml. Incubation time, 2 hrs. Table gives per cent of activity.

Iodoacetate M	Respiration	Glucose utilization	Lactate formation
—	100	100	100
1×10^{-3}	17	0	0
1×10^{-4}	42	37	6
1×10^{-5}	89	67	49
1×10^{-6}	100	100	80

In rabbit cerebral cortex slices there was a parallel inhibition of respiration and aerobic glycolysis. In every instance the concentrations of iodoacetate which decreased or abolished glucose utilization and lactic acid formation, ultimately

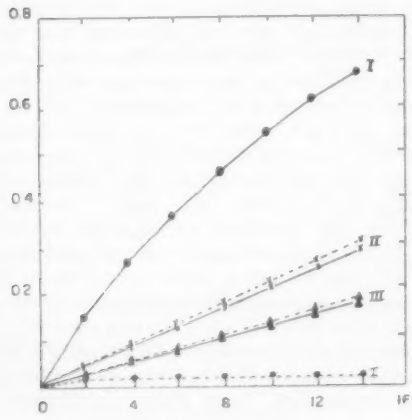

FIG. 1. — *EFFECT OF IODOACETATE ON THE ACTIVITY OF ENZYMES FOR GLUCOSE METABOLISM IN CELL-FREE EXTRACTS OF RABBIT CEREBRAL CORTEX. System according to ref. 19. Abscissa, time in minutes. Ordinate, O. D. at 340 mμ. Full line, control. Broken line, system with 0.001 M iodoacetate. I, phosphoglyceraldehyde dehydrogenase. II, phosphogluconic acid dehydrogenase. III, glucose-6-phosphate dehydrogenase.*

O. D.: Optical density.

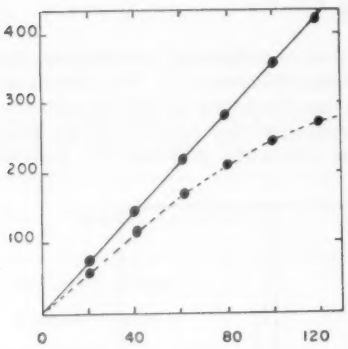

FIG. 2. — *EFFECT OF IODOACETATE ON THE OXIDATION OF PYRUVIC ACID BY RABBIT CEREBRAL CORTEX SLICES. Conditions similar to those described in Table II. Ordinate, oxygen uptake, μl. Abscissa, time in minutes. Full line, control. Broken line, system with 0.001 M iodoacetate.*

diminished respiration also. Lactate formation was more affected by iodoacetic acid than glucose utilization; respiration was the more resistant to this inhibitor. A residual respiration was observed even at a concentration at which glycolysis was completely abolished (Table II).

To find out if 0.001 M iodoacetate was specifically inhibiting phosphoglyceraldehyde dehydrogenase without producing non-specific toxic changes, we studied the effect of this same concentration on the enzymes of the pentose phosphate pathway. We found, in cell-free extracts of rabbit cerebral cortex, that the activity of glucose 6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase and the enzymes for ribose 5-phosphate breakdown, were the same in the presence or in the absence of iodoacetate; the formation of fructose and sedoheptulose from ribose 5-phosphate was not affected either, but the activity of phosphoglyceraldehyde dehydrogenase was completely abolished (Fig. 1 and Table III). These results may be interpreted as an indication that rabbit cerebral cortex utilizes almost exclusively the Embden-Meyerhof glycolytic pathway, and that the pentose phosphate shunt is not used appreciably even when glycolysis is completely inhibited. The residual respiration mentioned above must be due to oxidation of residual lactate or of other substrates within the cell which are not affected by iodoacetate. To find some support for this interpretation we incubated rabbit cerebral cortex slices with pyruvic acid and measured oxygen uptake; when incubations were carried out in the presence of 0.001 M iodoacetate, oxygen uptake was only slightly decreased (Fig. 2).

Some Intermediates of Glucose Metabolism

On incubation of rabbit cerebral cortex homogenates in the presence of glucose, neither ribose, nor sedoheptulose formation was detected, which may be an indication that the pentose phosphate pathway is probably not operating in this tissue; however, some intermediates of glucose metabolism do not accumulate and therefore, their absence cannot be taken as proof against the operation of the pathway to which they belong. The ether extract of the protein-free supernatant contained lactic acid, acetic acid and small amounts of other acids not as yet identified (Fig. 3). We had reported acetic acid accumulation in other normal and tumor cells (31), and more detailed work is actually under way.

Utilization of Labeled Glucose

The relative importance of the pathways in carbohydrate metabolism has also been estimated by the use of labeled glucose. Bloom et al. (25) postulated that the metabolism of glucose via the Embden-Meyerhof glycolytic pathway could be anticipated to result in the simultaneous contributions of carbon atom 1 and 6 to carbon dioxide. By an alternative oxidative pathway via pentose

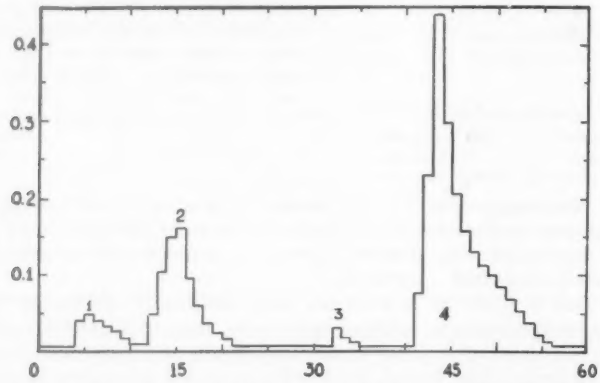

FIG. 3.—*COLUMN CHROMATOGRAM OF ORGANIC ACIDS FORMED FROM GLUCOSE BY RABBIT CEREBRAL CORTEX SLICES. Abscissa, tube number (3.5 ml of effluent fluid/tube). Ordinate, ml of 0.01 N NaOH; 2, acetic acid; 4, lactic acid; 1 and 3, unidentified. Effluent fluid, chloroform and butanol according to Bulen et al.* (14).

phosphate shunt, the appearance of C-1 as carbon dioxide would precede that of C-6. Many of the published estimates have been based on this relative incorporation of C^{14} from variously labeled glucose into respiratory CO_2 (26, 27, 28, 29). Although this procedure is capable of providing information about the pathways operating in some normal tissues, in other normal and tumor cells the CO_2 probably goes through many side reactions and the quantitative contributions made by the different pathways cannot be estimated by the ratio [$C^{14}O_2$ from glucose 6-C^{14}/$C^{14}O_2$ from glucose 1-C^{14}] (30, 31, 32). As a consequence it has been necessary to make assumptions, the validity of which are uncertain. The shortcomings of this means of estimation are discussed by Wood (33).

Blumenthal et al. (34) originated another procedure to estimate quantitatively the pathways of glucose metabolism of cells *in vitro*. The method is based on the fact that the symmetrical cleavage of the glucose carbon chain via the glycolytic pathway yields two C3 units. In this way any lactate derived from glucose-1-C^{14} or glucose-6-C^{14} should have one of six carbons labeled, and its specific activity would therefore be one-sixth that of the labeled carbon of glucose or the same (1.0 times) as the over-all specific activity of the glucose-1-C^{14} or glucose-6-C^{14}. On the other hand, if the pentose phosphate pathway is operating, carbon 1 of glucose is lost by decarboxylation of 6-phosphogluconate. Therefore, any lactate derived from glucose-1-C^{14} should be unlabeled and that derived from glucose-6-C^{14} would have a specific activity one-third that of the

TABLE III

The metabolism of ribose-5-phosphate by rabbit cerebral cortex homogenates in the presence and in the absence of 0.001 M iodoacetate.

System. — Homogenate 1 ml. Tris buffer 0.1 M pH 7.4, 1 ml. $MgCl_2$ 0.1 M, 0.5 ml. Ribose-5-phosphate, 15μM. Total vol., 3 ml. Temp. 38° C. Incubation time, 1 hr. Gas phase, air.

Measurements	Control μM	Iodoacetic acid 0.001 M μM
Ribose-5-P utilization	3.8	4.0
Fructose formation	0.6	0.6
Sedoheptulose formation	0.2	0.2

labeled carbon of glucose or two times that of the over-all activity of glucose-6-C^{14}, provided the C2 unit does not contribute to lactic acid formation. If the glycolytic pathway and the pentose phosphate shunt are operating simultaneously, the specific activity of a C3 unit should be less than 1.0 if it comes from glucose-1-C^{14} and greater than 1.0 if it is derived from glucose-6-C^{14}. There are some factors which complicate this way of estimation, one of which is endogenous dilution; Blumenthal et al. (34) have attempted to correct this with the aid of a simultaneous experiment with glucose-U-C^{14}. It is also assumed that

TABLE IV

Metabolism of labeled glucose by rabbit cerebral cortex slices

Cerebral cortex slices were incubated with 100μM of C^{14} labeled glucose in Ringer-phosphate as indicated in methods. Gas phase, O_2. Temp. 38° C. Total volume, 11.5 ml. Incubation time, 2 hrs.

Expt.	Labeled glucose	Glucose used μM	Lactic acid formed μM	CO_2 evol. μM	% counts/min. CO_2 + Lactate	% counts/min. CO_2
I	1-C^{14}		25.5	20.7		
	6-C^{14}		25.5	17.0		
	U-6^{14}		25.7	16.0		
II	1-C^{14}	17.0	29.2	39.5	82	14
	6-C^{14}	19.4	31.1	42.3	70	11
	U-6^{14}	17.5	25.1	38.6	70	19
III	1-C^{14}	22.0	26.2	29.0		8
	6-C^{14}	25.0	26.0	25.0		6
	U-6^{14}	15.7	19.0	22.0		18
IV	1-C^{14}	12.0	15.0	28.5		11
	6-C^{14}	13.0	22.6	33.5		10
	U-6^{14}	18.0	21.0	25.3		17
V	1-C^{14}	13.5	28.5	34.0	75	14
	6-C^{14}	17.5	27.8	32.3	71	12
	U-6^{14}	18.0	25.0	31.6	76	18

triose phosphate isomerase brings dihydroxyacetone phosphate and glyceraldehyde phosphate to complete isotopic equivalence and that there is no accumulation of pentose phosphate pathway intermediates. The calculations for lactate or any other C3 unit are made as follows: Relative specific activity (R. S. A.) = Sp. act of lactate/Sp. act. of glucose. Corrected R. S. A. = Observed R. S. A. for lactate from glucose-1-C^{14} or glucose-6-C^{14} / Observed R. S. A. for lactate from glucose-U-C^{14}

% lactate via glycolysis = R. S. A. times 100.

The authors of this method, calculated in the same way the relative specific activity and corrected relative specific activity for $C^{14}O_2$. If the $C^{14}O_2$

TABLE V

Specific activity of $C^{14}O_2$ produced from labeled glucose by rabbit cerebral cortex slices

		$C^{14}O_2$	
Expt.	**Labeled glucose**	**R. S. A.**	**Corr. R. S. A.**
II	1-C^{14}	0.38	0.69
	6-C^{14}	0.31	0.56
	U-C^{14}	0.55	
III	1-C^{14}	0.38	0.51
	6-C^{14}	0.36	0.48
	U-C^{14}	0.75	
IV	1-C^{14}	0.27	0.50
	6-C^{14}	0.24	0.45
	U-C^{14}	0.54	
V	1-C^{14}	0.43	0.68
	6-C^{14}	0.40	0.63
	U-C^{14}	0.63	

(R. S. A.) from glucose-1-C^{14} is greater than that recovered from glucose-6-^{14} or glucose-U-C^{14} it would indicate a preferential oxidation of carbon 1 of glucose which occurs in the pentose phosphate pathway. On the other hand, if the Embden-Meyerhof process is the main pathway, the R. S. A. of $C^{14}O_2$ from glucose-U-C^{14} would be greater than that recovered from glucose-1-C^{14}.

Rabbit cerebral cortex slices were incubated aerobically with glucose-1-C^{14}, glucose-6-C^{14} and glucose-U-C^{14}, and the amount of glucose utilized, lactic acid formed, and CO_2 absorbed and evolved, were measured. The total counts per minute in CO_2 and lactate were also determined (Table IV). Total recovery in CO_2 plus lactate ranged from 70 to 82 %, therefore 18 to 30 % of the C^{14} glucose metabolized was unaccounted for. The higher per cent of $C^{14}O_2$ from glucose-U-C^{14} clearly indicates that glucose carbon 1 is less rapidly oxidized than other glucose carbons. This finding is in harmony with the Embden-Meyerhof pathway, since in this process carbons 1 and 6 of glucose, which form the methyl carbon of lactate or acetate, is less rapidly oxidized than carbons 3 and 4 or carbons 2 and 5. On the other hand, when incubations were carried out with glucose-1-C^{14}, the per cent of $C^{14}O_2$ recovered was slightly

greater than that recovered from glucose-6-C^{14}. If it is assumed that the $C^{14}O_2$ from glucose-6-C^{14} is the amount contributed by the citric acid cycle, it may be calculated by difference, that only one to three per cent of the glucose used is oxidized through the pentose phosphate pathway. Thus, in rabbit cerebral cortex, the Embden-Meyerhof process seems to operate almost exclusively. A similar conclusion can be derived from the R. S. A. and corrected R. S. A. of $C^{14}O_2$ calculated according to Blumenthal et al. (34), (Table V). The R. S. A. for lactate as well as the corrected R. S. A. were calculated in the same way. The lactate derived from glucose-1-C^{14} and that from glucose-6-C^{14} had a corrected R. S. A. close to 1.0 (Table VI), indicating that lactic acid was formed

TABLE VI

Specific activity of lactic acid formed aerobically from labeled glucose by rabbit cerebral cortex slices

Conditions similar to those described in Table IV.

	Glucose-1-C^{14}		Glucose-6-C^{14}		Glucose-U-C^{14}	
	II	V	II	V	II	V
Glucose, R. S. A.	1.0	1.0	1.0	1.0	1.0	1.0
Lactic acid, R. S. A.	0.79	0.72	0.74	0.62	0.71	0.64
Lactic acid, corr. R. S. A.	1.10	1.09	1.03	0.97		
Lactate via Embden-Meyerhof pathway	Approx. 100		100			

Approx.: Approximately.

almost exclusively via the glycolytic pathway. The value for lactate from glucose-1-C^{14} was slightly greater than 1.0; this is probably an experimental error, otherwise it would indicate a preferential oxidation of carbon 6 of glucose as occurs in the uronic acid pathway. However the corrected R. S. A. for lactate from glucose-6-C^{14} which is around 1.0 does not support this point of view, and neither does our finding that glucose carbon 1 is slightly more oxidized over glucose carbon 6. Preliminary results reported by Wenner et al. (30) using glucose-1-C^{14} and glucose-6-C^{14} in rat brain are in agreement with our findings.

As another check on the occurrence of the oxidative pentose phosphate pathway, glucose-2-C^{14} was used to measure the specific activities of the three carbon atoms in the lactic acid produced. Glucose metabolized through this pathway would produce pentose phosphate-1-C^{14} and through the transketolase transaldolase reactions, hexose monophosphate with 74 % of C^{14} in carbon 1 and 24 % in carbon 3 according to Horecker et al. (35); this would produce lactate with carbon 3 more heavily labeled than carbon 1. On degradation of lactic acid, about 99 % of the total activity was found in carbon 2 (Table VII), which may be another indication of an almost exclusive operation of the Embden-Meyerhof pathway.

It is actually known that the pentose phosphate pathway can operate nonoxidatively by transketolation of fructose-6-phosphate (1, 36). If it occurs in

brain, the calculations based on the specific activities of lactic acid would be invalidated. However this mechanism has not yet been explored in this tissue. According to Horecker and Hiatt (1), evidence is accumulating that the pentose phosphate pathway through its ability to produce reduced triphospho-

TABLE VII

Degradation of lactic acid formed from glucose-2-C14 by rabbit cerebral cortex slices

Conditions as in Table IV.

Carbon atom	Sp. activity Counts/min./μM	Total %
1	15.3	0.8
2	1,977.0	98.7
3	10.0	0.5

pyridine nucleotide (TPNH) would have an important function in cell growth and synthesis; some tissues such as liver and lactating mammary gland, which are actively synthesizing tissues, would utilize the pentose phosphate shunt to an appreciable extent. In cerebral cortex, we have obtained no evidence for an important participation of this pathway, therefore the Embden-Meyerhof process, which is almost exclusive in this tissue, may be utilized mainly for the breakdown of glucose and the production of energy. In liver, the glycolytic route, would serve primarily for gluconeogenesis.

Summary

Cell-free extracts from rabbit cerebral cortex contain enzymes for the metabolism of glucose via the Embden-Meyerhof glycolytic pathway and the pentose phosphate shunt.

The effect of graded concentration of iodoacetic acid on the oxygen consumption, glucose utilization and lactic acid formation by rabbit cerebral cortex slices were investigated. In every instance, if the glucose utilization or the lactate formation was inhibited, the oxygen consumption was inhibited also. On the other hand, at a concentration that abolished aerobic glycolysis, iodoacetate had no effect at all on the enzymes of the pentose phosphate pathway.

In order to obtain further information, tracer studies were carried out with variously labeled glucose. Estimations were based on three procedures: (1) the yield of $C^{14}O_2$; (2) the specific activity of lactate from glucose-1-C^{14} and glucose-6-C^{14}; and (3) the measurement of the specific activities of the three carbon atoms of the lactic acid produced from glucose-2-C^{14}. The results provide evidence that the pathway of glucose metabolism in rabbit cerebral cortex is almost exclusively via the Embden-Meyerhof glycolytic process.

References

(1) Horecker, B. L., Hiatt, H. H.: *New Engl. J. Med.*, 1958, *258*, 177-184, 225-232.
(2) Racker, E.: *Advanc. in Enzymol.*, 1954, *15*, 141.
(3) Barker, S. B., Shorr, E., Malam, M.: *J. biol. Chem.*, 1939, *129*, 33.
(4) Fuhrman, F. A., Field, J.: *J. cell. comp. Physiol.*, 1943, *21*, 307.
(5) McIlwain, H.: *Biochem. J.*, 1951, *49*, 382.
(6) Umbreit, W. W., Burris, R. H., Stauffer, J. F.: *Manometric Techniques and Tissue Metabolism*, Burgess Publishing Co., 1949.
(7) McIlwain, H.: *Biochemistry and the Central Nervous System*, Little Brown, 1955.
(8) Barker, S. B., Summerson, W. H.: *J. biol. Chem.*, 1941, *138*, 535.
(9) Keilin, D., Hartree, E. F.: *Biochem. J.*, 1945, *39*, 293.
(10) Ma, T. S., Zuazaga, G.: *Ind. Eng. Chem., Anal. Ed.*, 1942, *14*, 280.
(11) Mejbaum, W.: *Z. physiol. Chem.*, 1939, *258*, 117.
(12) Horecker, B. L., Smyrniotis, P. Z., Klenow, H.: *J. biol. Chem.*, 1953, *205*, 661.
(13) Roe, J. H.: *J biol. Chem.*, 1934, *107*, 15.
(14) Bulen, W. A., Varner, J. E., Burrell, R. C.: *Anal. Chem.*, 1952, *24*, 187.
(15) *Handbook of Isotopic Tracer Methods.* Edited by Sakami, W. Western Reserve University, School of Medicine, 1955.
(16) Van Slyke, D. D., Steele, R., Plazin, J.: *J. biol. Chem.*, 1951, *192*, 769.
(17) Neish, A. C.: *Analytical Methods for Bacterial Fermentations.* National Research Council of Canada, 1952.
(18) Van Slyke, D. D., Folch, J.: *J. biol. Chem.*, 1940, *136*, 509.
(19) Ghiretti, F., Barron, E. S. G.: *Biochem. et Biophys. Acta*, 1954, *15*, 445.
(20) Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M. L., Farr, A. L., Albers, R. N.: *J. biol. Chem.*, 1954, *207*, 39
(21) Buell, M., Lowry, O. H., Roberts, N. R., Chang, M., Kappan, J.: *J. biol. Chem.*, 1958, *232*, 979.
(22) Long, C.: *Biochem. J.*, 1952, *50*, 407.
(23) Glock, G. E., McLean, P.: *Biochem. J.*, 1954, *56*, 171.
(24) Lundsgaard, E.: *Biochem. Z.*, 1930, *217*, 162.
(25) Bloom, B., Stetten, D. W., Jr.: *J. Amer. chem. Soc.*, 1953, *75*, 5446.
(26) Bloom, B., Steette, M. R., Stetten, D. W., Jr.: *J. biol. Chem.*, 1953, *204*, 681.
(27) Katz, J. Abraham, S., Hill, R., Chaikoff, I. L.: *J. Amer. chem. Soc.*, 1954, *76*, 2277.
(28) Agranoff, B. B., Brady, R. O., Colodzin, M.: *J. biol. Chem.*, 1954, *211*, 773.
(29) Kinoshita, J. H., Watch, C.: *J. biol. Chem.*, 1958, *233*, 5.
(30) Wenner, C. E., Weinhouse, S.: *J. biol. Chem.*, 1956, *222*, 399.
(31) Villavicencio, M., Barron, E. S. G.: *Arch. Biochem. Biophys.*, 1957, *67*, 121.
(32) Kit, S.: *Cancer Res.*, 1956, *16*, 70.
(33) Wood, H. G.: *Physiol. Rev.*, 1955, *35*, 841.
(34) Blumenthal, H. J., Lewis, K. F., Weinhouse, S.: *J. Amer. chem. Soc.*, 1954, *76*, 6093.
(35) Horecker, B. L., Gibbs, M., Klenow, H., Smyrniotis, P. Z.: *J. biol. Chem.*, 1954, *207*, 393.
(36) Dickens, F.: *Ann. N. Y. Acad. Sci.*, 1958, *75*, 71.

INFLUENCE OF STEROID HORMONES ON THE RESPIRATORY SYSTEM OF MAMMALIAN MITOCHONDRIA (*)

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THE PRESENT investigation was undertaken to examine the effect of steroid hormones on the respiratory system of mammalian mitochondria. There have been a number of recent reports (1, 2) showing that steroid hormones such as β-estradiol can function as coenzymes in transhydrogenase reactions. These steroid – mediated transhydrogenase reactions have been demonstrated in purified enzymes, which are believed to be derived from soluble or microsomal origin. It was of interest, therefore, to examine the action of steroids on pyridine and non-pyridine nucleotide-linked dehydrogenase systems of intact phosphorylating mitochondria. It seemed possible that this approach migth help to clarify the role of steroids in metabolic control mechanisms of living cells.

METHODS

Rat heart mitochondria (sarcosomes) were prepared in sucrose isolation medium (+) according to the method of Cleland and Slater (3). Assays of respiration and phosphorylation were carried out in 2 ml volume in a medium containing sucrose (0.32 M), versene (0.1 mM), KCl (0.018 M), phosphate buffer (0.018 M at pH 7.5), sarcosomes (approximately 1 mg Protein); the other additions which are indicated in figures and tables.

Rat liver mitochondria were prepared according to the method of Schneider and Hogeboom (4) in 0.25 M sucrose. The assay medium (2 ml) contained sucrose (0.25 M), KCl (0.04 M), $MgCl_2$ (0.025 M), phosphate buffer (0.02 M at pH 7.5), and liver mitochondria (approximately 1 mg Protein).

Respiration and oxidative phosphorylation was assayed polarographically

(*) This investigation was carried out while on leave of absence from Divisão de Patologia Experimental, Instituto de Biologia e Pesquisas Tecnológicas, Caixa Postal, 357, Curitiba, Estado do Paraná, Brasil. This investigation was supported by grants from The Rockefeller Foundation and from Conselho Nacional de Pesquisas (Brasil).

(+) Sucrose (0.32 M) – versene (0.001 M adjusted to pH 7.5 with NaOH).

by the method described by Chance and Williams (5) employing an oxygen electrode apparatus of the type suggested by Davies and Brink (6).

Changes in the oxidation-reduction level of the respiratory enzymes were followed using the double-beam spectrophotometer described by Chance (7). This instrument records the arithmetic difference in light absorption between two wavelengths which pass through a single cuvette. Changes in oxidation-reduction state of cytochrome *b* were followed at 430 mμ minus 410 mμ; the former wavelength being the region where cytochrome *b* specifically absorbs and the latter being a nearby reference wavelength used to compensate for non-specific changes in light absorption in the dense mitochondrial suspensions.

The effect of diethylstilbestrol on mitochondrial structure was examined by measuring light-scattering at 410 mμ. This technique was first introduced by Cleland (8) and Raaflaub (9) for examination of swelling and shrinking changes in mitochondrial suspensions.

Results

A typical experiment showing the techniques employed for assay of respiration and oxidative phosphorylation is shown in figure 1. The experiment was begun (on the left) by the addition of an aliquot of a mitochondrial suspen-

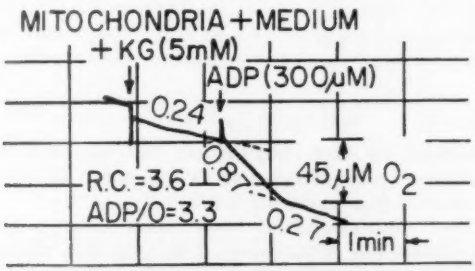

FIG. 1. — *Polarographic assay of respiration and oxidative phosphorylation in rat heart mitochondria.*

sion and the oxidizable substrate, α-ketoglutarate (KG), to the air-saturated medium (240 μM O_2). An abrupt drop in the level of the tracing indicates that the oxygen concentration of the suspending medium has been diluted by the mitochondrial suspension, whose concentration of dissolved oxygen is less than air saturated. The downward deflection of the curve indicates the rate of oxygen consumption; these rates were calculated in μ-moles O_2 consumed per second per liter and are written below the tracing. α-KG caused a slow constant rate of respiration characteristic of mitochondria in the quiescent state 4 (10). On addition of 300 μM adenosine diphosphate (ADP), respiration was augmented 3.6-fold; this accelerated phase of respiration is known as the active state or state 3. Approximately 1 minute following the addition of ADP, respiration declined by a factor of 3.3-fold, and the mitochondria are once again in the quiescent state. The respiratory control (R. C.) demonstrated by this experiment shows that the mitochondria are "tightly-coupled", i. e., res-

piration is accelerated by addition of the phosphate-acceptor, ADP, and returns to its initial rate of substrate respiration when ADP is expended due to oxidative phosphorylation.

The ADP: oxygen ratio in "tightly-coupled" preparations is equivalent to the phosphorous: oxygen ratio since the equilibrium of the reaction,

$$ADP + P \rightleftharpoons ATP \qquad (I)$$

is far to the right. In figure 1, this value is calculated from the amount of ADP added (300 μM) and the amount of O_2 utilized (45 μM or 90 μ atoms

TABLE I

Compound	Conc. μM	Respiration μM O_2/sec	Respiratory control	ADP/O	% increase in state 4 respiration
None	—	0.23	6.2	2.97	0
Cortisone acetate	100	0.23	5.4	2.97	0
17-Hydroxycorticosterone	250	0.23	4.6	2.80	11
Desoxycorticosterone acetate	400	0.17	3.8	2.80	0
Desoxycorticosterone	200	0.19	2.5	2.80	0
None	—	0.14	3.4	3.33	0
Testosterone	100	0.14	2.3	2.78	62
Pregnanediol	100	0.13	1.3	—	—
Progesterone	100	0.13	2.4	2.43	54
Diethyl-Stilbestrol	50	0.10	2.1	2.45	69
B-Estradiol	150	0.13	2.5	2.86	36

per liter) for conversion of ADP to ATP, viz. $300/90 = 3.3$. This value for the efficiency of oxidative phosphorylation is in good agreement with earlier studies (11) of mitochondria oxidizing α-KG.

"Loosely-coupled" mitochondrial suspensions show poor respiratory control, namely, respiration can be augmented by addition of phosphate acceptor, but the latter is either not expended or only partially expended. Under these conditions, respiration will not return to that of the quiescent state. One explanation for this phenomenon is increased ATP-ase activity, and when this occurs the equilibrium of equation I is shifted to the left (11).

Table I summarizes the effect of steroids on the respiration of α-KG by heart sarcosomes. The data were obtained by the procedure described in figure 1. At the concentrations examined in this series of experiments, representative species of adrenal steroids, estrogens, androgens, and progesterones do not markedly affect the rate of α-KG respiration in heart mitochondria. The concentrations employed were selected from titration curves to be in a range where their addition initially caused little or no change in respiration. Higher concentrations of steroids progressively inhibited respiration while lower concentrations have no apparent effect on mitochondria. In the concentration range shown in table I, only pregnandiol and desoxycorticosterone significantly lowered the respiratory stimulation ratio and in the former case this was sufficiently marked to abolish phosphorylation. With the other steroids the efficiency of oxidative phosphorylation was not very different from the control values although the trend was to lower the ADP/O values.

Following the exhaustion of ADP in the experiments summarized in table I, it was observed that with some of the steroids, the rate of respiration did not return to the initial rate obtained on α-KG (i. e. showed poor respiratory control). These findings are summarized in the last column where the percentage increase over the initial rate of α-KG respiration is given. Compounds of the androgen and progesterone group showed a considerable elevation over controls in this regard. This finding is quite similar to results obtained with "loosely-coupled" mitochondria as described above, and would explain the trend toward slightly lower ADP/O ratios in the presence of these steroids. Diethylstilbestrol, a synthetic steroid, was most efficient in this regard, increasing the state 4 respiration by 69 % even though the concentration of the compound was smaller than any of the other compounds tested. These results suggest an increased ATP-ase activity in the presence of certain steroids. Maley and Johnson [12] have demonstrated that a definitive relationship may exist between ATP-ase activity of mitochondria and their morphological integrity. These investigators showed that a number of detergents and uncouplers of oxidative phosphorylation could increase ATP-ase activity. In the case of detergents a disruption of the mitochondria could be brought about, and they suggested that in this case, the augmented ATP-ase activity was a result of structural changes [12]. Their conclusions are consistent with earlier work of Potter, Siekowitz and Simonsen [13] who showed that the activation of a latent ATP-ase in mitochondria could be correlated with the swelling of mitochondria on ageing. The results of table I indicate that these findings [12, 13] must be considered in regard to the effects of steroids on the respiratory system of mitochondria.

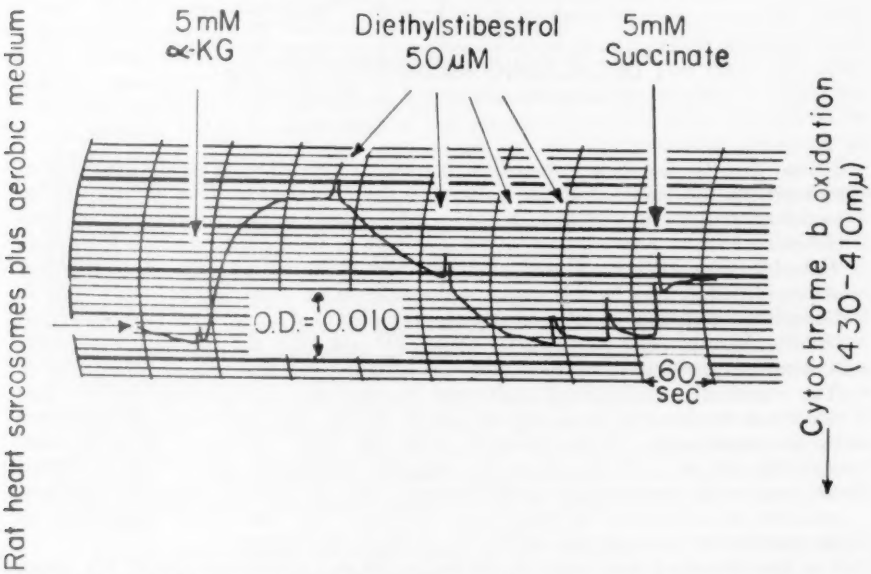

FIG. 2. — *Effect of Diethylstilbestrol on steady —state oxidation— reduction level of cytochrome b.*

It seemed possible that the inhibitory action of steroids on respiration (α-KG) at higher concentrations than employed in table I may have some relationship to their effect on structural integrity of mitochondria as suggested by augmented ATP-ase activity (as judged by state 4 respiration) at intermediate concentrations.

Diethylstilbestrol (DES), the synthetic steroid which markedly affect state 4 respiration was further examined. Higher concentrations were used to study its effect on the steady state of the respiratory enzymes in an effort to locate the site of inhibition of respiration. Figure 2 shows such an experiment with rat heart sarcosomes. The experiment was begun (on the left) by the addition of a concentrated mitochondrial suspension to an aerobic medium. A slow oxidation of cytochrome *b* to a new steady-state is observed under these con-

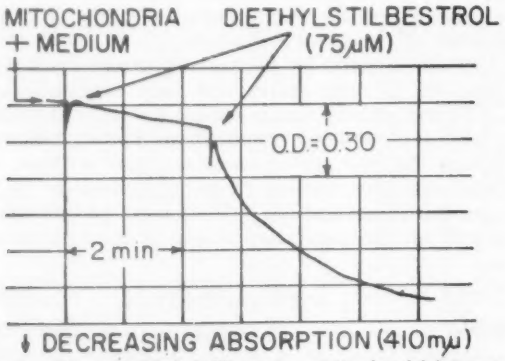

FIG. 3. — *Effect of Diethylstilbestrol on Mitochondrial Structure.*

ditions due to the slow exhaustion through respiration of small amounts of endogenous substrate which are present in the preparation. The addition of an excess of exogenous substrate, 5 mM α-KG, caused a rapid reduction of cytochrome *b* to a new steady-state (state 4) which is completed in about 1 minute. Titration with DES in 50 μM additions was then carried out; the inhibition of respiration caused by this compound results in an oxidation of cytochrome *b*. Complete oxidation of cytochrome *b* was obtained at a final concentration of 100 μM. Although in the presence of 200 μM DES the respiration of α-KG is completely inhibited, addition of 5 mM succinate can still reduce cytochrome *b*. The extent of cytochrome *b* reduction by succinate is, however, smaller than which can be obtained in the presence of the steroid. Two points are demonstrated by this experiment. First, transition from the slight inhibition of respiration with 50 μM DES (Table I) to the complete inhibition shown here, occurred within a small concentration range. Secondly, the fact that cytochrome *b* becomes oxidized on inhibition of respiration indicates that the site of inhibition of the respiratory chain occurs below the level of cytochrome *b*.

To determine the point of inhibition in the respiratory chain, the effect

TABLE II

Effect of steroid compounds on loss of 340 mμ absorbing substance from mitochondria

The reaction mixture (3.0 ml.) contained glycylglycine buffer (0.16 M at pH 7.5), $MgCl_2$ (0.008 M), NaF (0.008 M), Sodium phosphate buffer (0.01 M at pH 7.5), sucrose (0.03 M, B-hydroxybutyrate (0.005 M), ADP (0.15 mM), rat liver mitochondria (1.0 mg. Protein) and the other additions shown in the table. The reaction mixture was incubated for 30 minutes at 25° C, and then centrifuged at 10 000 x G for 10 minutes. The mitochondria-free supernatant was decanted and the optical density read in the Beckman Spectrophotometer.

Compound	Optical density
Control	0.045
Ethanol control	0.038
Diethyl stilbestrol (100 μM)	0.085
B-Estradiol (100 μM)	0.070
Chen C-62 (*) (100 μM)	0.094

(*) A synthetic compound kindly donated by Dr. C. Chen, Northwestern University, Chicago, Illinois.

of 100 μM DES on the steady-state of flavin and pyridine nucleotides, was examined. Like cytochrome *b*, flavin oxidation (465-510 mμ measurement) during α-KG oxidation was brought about by steroid addition. However, DES addition caused no change in the oxidation-reduction level of pyridine nucleotide (340-374 mμ measurement), under similar conditions. These results suggested that the site of inhibition of respiration was at the dehydrogenase level.

Mitochondria which are damaged by physical and chemical methods have been reported to lose bound pyridine nucleotide [14, 17]. Therefore, the effect of DES and some other steroids on leakage of materials which absorb at 340 mμ was studied. Table II shows that in the presence of either DES, β-estradiol, or another synthetic steroid, an increase in the loss of material absorbing at 340 mμ from mitochondria occurred.

Furthermore, when the effect of DES on mitochondrial structure was examined by measuring the turbidity or light-scattering of a mitochondrial suspension, it was found that the mitochondria undergo virtual dissolution (figure 3). Addition of 75 μM DES initiated the swelling of mitochondria as shown by the decreasing turbidity of the suspension. A second 75 μM addition of steroid caused dissolution of the mitochondria. These effects are wavelength-independent. Apparently when a critical concentration of steroid is reached, a disruption of the mitochondria occurs. This finding would explain the sudden transition between little to complete inhibition of respiration within a narrow concentration range noted earlier. Although the experiment reported in table II indicates that the loss of material absorbing at 340 mμ (presumably pyridine nucleotide) is not as extensive as one might imagine, it is quite likely under these circumstances that pyridine nucleotide no longer exists in a form bound to protein.

Chance and Conrad [18] have reported that the addition of synthetic detergents to mitochondrial suspensions such as desoxycholate results in loss of fluorescence attributable to bound pyridine nucleotide.

Discussion

The influence of steroids and steroid hormones on the respiratory system of mammalian mitochondria is a concentration dependent phenomenon. The action of these compounds on mitochondria appear to fall within three concentration ranges.

1) In the lowest concentration range, addition of steroids to mitochondria respiring α-KG caused no observable change in respiration, the respiratory control ratio, or the efficiency of oxidative phosphorylation.

2) In the highest concentration range, steroid addition completely inhibited α-KG respiration and largely abolished succinate respiration. Neither respiratory control ratio nor oxidative phosphorylation was detected. Spectrophotometric experiments with DES showed that virtual dissolution of the mitochondria can occur.

3) In an intermediate concentration range, selected to be at the borderline of inhibition of respiration, the respiratory control ratio, and efficiency of oxidative phosphorylation, tended to be slightly lowered. After completion of a short cycle of oxidative phosphorylation, the rate of α-KG respiration in the presence of some steroids was increased over that obtained initially on α-KG, suggesting ATP-ase activity. Spectrophotometric experiments indicated that structural changes (swelling) in the mitochondrial suspension was occurring.

The above-mentioned findings appear consistent with the conclusion that the action of steroids and steroid hormones on mitochondrial respiratory processes are the consequence of a steroid-induced swelling, i. e., a change in mitochondrial structure.

In experiments designed to observe the effect of mitochondrial swelling on function, it has been often observed [14, 17] that pyridine nucleotides are readily lost by mitochondria and that the ability to oxidize pyridine nucleotide-linked substrates correspondingly declines. The respiration of succinate, which enters the respiratory chain at the level of cytochrome *b*, is generally maintained in the initial stages of swelling, but this activity also declines as the swelling process continues. The capacity to carry out oxidative phosphorylation is lost under these circumstances, and if swelling has not proceeded too far along, can be restored by addition of high concentrations of diphosphopyridine nucleotide. These considerations would appear to explain the inhibitory action of DES at high concentrations noted here, and the reversibility by diphosphopyridine nucleotide of glutamic dehydrogenase activity in DES-treated liver mitochondria, observed by Emmlot and Bos [19].

A positive qualitative correlation between mitochondrial swelling induced by synthetic detergents and ATP-ase activity has been reported by Witter and Mink [20] and Witter and Cottone [21]. Witter and Mink [20] have shown that it is specifically the magnesium rather than the dinitrophenol-activated ATP-ase which increases activity on treatment of mitochondria with detergents. In agreement with these results, other investigators have demonstrated [22] that ageing of mitochondrial suspensions is accompanied by increases in magnesium-activated ATP-ase activity. These findings are consistent with the increased rate of state 4 respiration in the presence of steroids and intramitochondrial ATP (from oxidative phosphorylation) noted in Table I. Indeed, similar findings were obtained in an earlier study by activating ATP-ase activity by directly adding magnesium ions to suspensions of heart mitochondria respiring

α-KG (11). Thus it appears that within a narrow concentration range (intermediate) steroids and steroid hormones can stimulate respiration as a result of increased ATP-ase activity, the latter being brought about by mitochondrial swelling.

The ability of steroids to disrupt mitochondria by their detergent properties would explain why the transition from no effect on respiration (α-KG) to complete inhibition occurs within a small change in concentration. The results of figure 3, are compatible with the idea that when a critical concentration of steroid is reached, sudden, rapid dissolution of mitochondrial structure will occur.

Since the results of the present investigation are consistent with the idea that steroids affect mitochondrial structure as a result of their ability to act as detergents, it is difficult to ascribe these results to the biological activity of steroid hormones. Steroid hormones are highly specific in their action, whereas in the mitochondrial experiments low specificity is demonstrated. It would appear coincidental that steroids can affect the function of the respiratory chain at the dehydrogenase level, this in view of their highly specific participation in dehydrogenase activity in enzymes from the soluble and microsomal portions of the cell. (1, 2). It is, however, possible that steroid hormones could function in the control of metabolic reactions by altering the permeability of membranes, as the mitochondrial membrane.

It is of interest that the status of another hormone, thyroxine, which is presently obscure, bears some similarity to the questions under consideration here (23). Thyroxine can cause mitochondrial swelling, can augment ATP-ase activity, and increases the rate of respiration in systems at higher levels of integration than mitochondria, such as tissue slices, perfused organs and intact animals. Similarly, at appropriate concentrations, the addition of steroid hormones are accompanied by increases in ATP-ase activity and respiration, and in mitochondrial swelling. At higher levels of integration steroid hormones have been shown to augment metabolism in surviving tissue slices (24), liver perfusion systems (25) and in the intact organism (26). The effect of steroids in this regard appears to be restricted to a narrow concentration range; with thyroxine and some of its derivatives, a broad concentration range can be employed. Although thyroxine and its derivatives cause a concentration dependent mitochondrial swelling, these agents do not disrupt mitochondria as readily as steroids, thus the latter agents are more inhibitory.

Definitive evidence is unavailable at the present time to make a decision concerning the role of thyroid and steroid hormones in the control of metabolic reactions by virtue of their ability to react with biological membranes. It does, however, seem appropriate to point out that, in studies conducted in organized biological systems, the ability of steroids and steroid hormones to cause mitochondrial swelling must be considered in evaluating their action.

Summary

The influence of several steroids and steroid hormones on the respiratory system in mammalian mitochondria was investigated by monitoring respiration, the steady-state oxidation-reduction level of cytochrome *b*, and structural changes (turbidity) shown by mitochondrial suspensions. The concentration of steroid added to the mitochondrial suspension determined its subsequent effect. At low concentrations no change in respiration, the respiratory control ratio or

the efficiency of oxidative phosphorylation occurred. At high concentrations the steroids could completely inhibit respiration of α-ketoglutarate and partially inhibit succinate oxidation. The inhibition of respiration was correlated with structural changes which at high concentrations may involve the virtual dissolution of mitochondria.

At intermediate concentrations, selected to be just below the threshold for inhibition of respiration, the following results were noted: respiration was unchanged, oxidative phosphorylation and respiratory control ratios tended to be lowered. These results could be explained by increases in adenosine triphosphatase activity and changes in mitochondrial structure (swelling).

The above findings were discussed in relation to the specificity of action of steroids in biological systems.

References

(1) Talalay, P., Williams-Ashmann, H. G.: *Proc. nat. Acad. Sci.*, 1958, *44*, 15.
(2) Talalay, P., Hurlock, B., Williams-Ashman, H. G.: *Proc. nat. Acad. Sci.*, 1958, *44*, 862.
(3) Cleland, K. W., Slater, E. C.: *Biochem. J.*, 1953, *53*, 547.
(4) Schneider, W. C., Hogeboom, G. H.: *Cancer Res.*, 1951, *11*, 1.
(5) Chance, B., Williams, G. R.: *Nature* (Lond.), 1955, *175*, 1120.
(6) Davies, P. W., Brink, F., Jr.: *Rev. sci. Instrum.*, 1942, *13*, 524.
(7) Chance, B.: *Rev. sci. Instrum.*, 1951, *22*, 619.
(8) Cleland, K. W.: *Nature* (Lond.), 1952, *170*, 497.
(9) Raaflaub, J.: *Helv. physiol. pharmacol. Acta*, 1952, *11*, 142.
(10) Chance, B., Williams, G. R.: *Advanc. Enzymol.*, 1956, *17*, 65.
(11) Packer, L.: *Arch. Biochem. Biophys.*, 1957, *70*, 290.
(12) Maley, G. F., Johnson, D.: *Biochim. Biophys. Acta*, 1957, *26*, 522.
(13) Potter, V. R., Siekowitz, P., Simonsen, H. C.: *J. biol. Chem.*, 1953, *205*, 893.
(14) Ernster, L.: *Exp. Cell Res.*, 1956, *10*, 704.
(15) Ernster, L.: *Exp. Cell Res.*, 1956, *10*, 721.
(16) Ernster, L., Löw, H.: *Exp. Cell Res., Suppl.*, 1955, *3*, 133.
(17) Hunter, F. E., Jr.: *Proc. 3rd Intern. Cong. Biochem.*, Brussels, 1955, *3*, 298.
(18) Chance, B., Conrad, H.: *Fed. Proc.*, 1958, *17*, 200.
(19) Emmlot, P., Bos, C. J.: *Exp. Cell Res.*, 1958, *14*, 132.
(20) Witter, R. F., Mink, W.: *J. Biophys. Biochem. Cytol.*, 1958, *4*, 73.
(21) Witter, R. G., Cottone, M. A.: *Biochim. Biophys. Acta*, 1956, *22*, 372.
(22) Siekovitz, P., Löw, H., Ernster, L., Lindberg, O.: *Biochim. Biophys. Acta*, 1958, *29*, 378.
(23) Lehninger, A. L.: *Proceedings of the International Symposium on Enzyme Chemistry*, Tokyo, Pan-Pacific Press, P. 297, 1958.
(24) Pesch, L. A., Topper, Y. J.: *Biochim. Biophys. Acta*, 1958, *30*, 207.
(25) Carrol, N. V.: *Unpublished results.*

A STUDY OF THE CYTOCHROMES OF MARINE INVERTEBRATES (*)

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The study of cell respiration has established that the pathway to oxygen occurs through a chain of components between the substrate and oxygen. Whereas the principle of electron transfer appears to be unique, the components of the chain may vary from organism to organism. Because of the great differences among living organisms, it is of interest to know the nature of these components and to determine the extent and limitations of their similarities and differences.

As E. S. G. Barron pointed out in his studies on Comparative Biochemistry, if it is true that a fundamental unity exists in all forms of life in spite of any apparent evidence, it is also true that the machinery of metabolic processes is not the same in all organisms. A detailed analysis of the metabolic patterns and of the concerned enzymes is therefore necessary both for the explanation of the wonderful diversity existing in nature and for the understanding of the fundamental principles which make unity among diversity.

Experimental and results

The cytochrome system has been studied in the following groups of Marine Invertebrates: Cephalopods, Gastropods, Echinoderms and Anellids. Methods of preparation of isolated particles (in some cases identified as mitochondria and microsomes) from different tissues have been worked out. The presence of a cytochrome system in the particles has been investigated; the components have been identified and their enzymatic activities determined; some of the pigments have been extracted, purified and their chemical properties compared with those of mammalian cytochromes. Finally the rôle of the cytochrome system in respiration of Marine Invertebrates has been demonstrated in view of the possible participation of other enzymatic systems.

(*) Supported by a grant (RG-4845 C) of the United States Public Health Service. An abstract was presented to the IV Intern. Congress of Biochemistry, Vienna, 1958.

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1. *The cytochrome system of Cephalopods*

Hemocyanin is the oxygen carrier pigment of the blood of Cephalopods. It has been suggested that these animals might also have copper oxidases for terminal respiration. By manometric experiments we have demonstrated that tissue respiration in Cephalopods is inhibited by cyanide and carbon monoxide in the dark. The carbon monoxide inhibition of respiration is totally relieved by light. No participation of copper enzymes could be demonstrated. It seems therefore that the pathway of terminal respiration in Cephalopods is catalysed by iron porphyrin enzymes (1).

Mitochondria were prepared from the body muscle and from several organs of *Octopus vulgaris* (2). Sucrose at any concentration was found to be unsuitable. A suspension of particles was obtained by homogenization of the frozen tissue in cold 0.1 M phosphate buffer pH 7.3 and by differential centrifugation followed by repeated washing. Microscopic observation of the particles and the color reactions with vital dyes are consistent with the behaviour of mitochondria prepared from mammalian tissues. Difference spectra (the difference in optical density between the reduced and the steady-state oxidized forms of the pigments present) were taken after reduction by addition in the absence of air (gas phase hydrogen) of either sodium succinate, DPNH, ascorbate or dithionite. At the long wave end of the spectrum the α band of cytochrome *a* at 605 mμ is observed. The band at 560 mμ is the α band of cytochrome *b* whereas the fairly sharp peak at 554 mμ can be attributed to cytochrome c_1. The β bands of cytochrones *b* and *c* are in the region of 530 to 520 mμ. A trough at 470 mμ is due to disappearance of the absorption band of oxidized flavoproteins, whereas in the Soret region the distinctive band at 444 mμ is due to cytochrome a_3. The Soret band of cytochrome *b* is seen at 430 mμ. The detection of distinct bands corresponding to cytochrome *c* is rather difficult. This water soluble hemochromogen probably had been washed out during the preparation of the particles. Carbon monoxide, which is the most specific reagent for the study of terminal oxidases, was used for complete identification of cytochrome a_3. A sharp absorption band having a peak at 430 mμ and a trough at 445 mμ was clearly observed, both characteristic of the carbon monoxide compound of cytochrome a_3.

2. *The cytochrome c from the hepatopancreas of Sepia*

Direct evidence for the presence of cytochrome *c* in Cephalopods was obtained by extraction and purification of the pigment from the hepatopancreas of *Sepia officinalis* (3). The pigment shows in the reduced state maxima at 416 mμ, 520 mμ and 550 mμ, and, like mammalian cytochrome *c*, does not form a pyridine hemochromogen at alkaline pH, and the prosthetic group is not split and dissolved by acidic acetone. However, the cytochrome *c* extracted from *Sepia* hepatopancreas has several peculiar properties. The pigment is destroyed by acidic extraction and the purification procedure is based on alcaline treatment. It precipitates between 50 and 70 % saturation of ammonium sulfate and is sedimented by prolonged centrifugation at 105 000 x g. It is destroyed by heating to 80° C for 2 min and is not oxidized by mammalian cytochrome oxidase preparation.

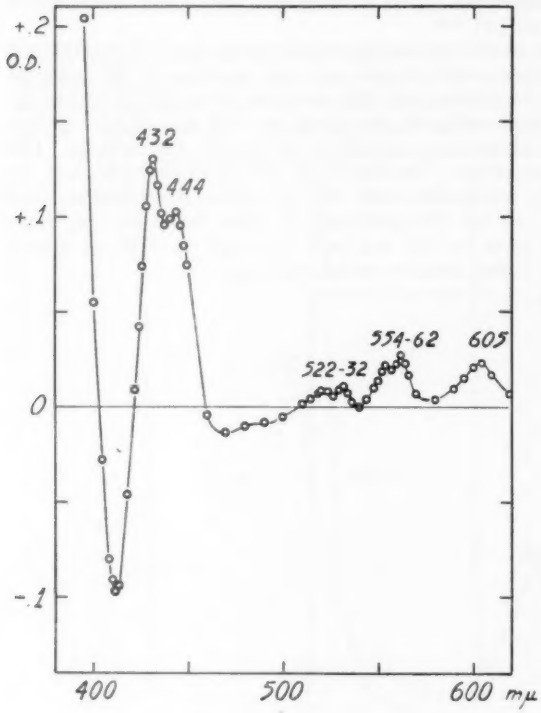

FIG. 1. — *APLYSIA LIMACINA. Buccal muscle particle preparation N° 10. Difference spectrum after reduction with DPNH.*

3. *The cytochrome system of Gastropods*

Aplysia is an herbivorous marine Gastropod which has no oxygen carrier pigment in the blood. This fact, together with the presence of large amounts of myoglobin in muscles, makes this animal interesting from the point of view of the Comparative Biochemistry of the respiratory enzymes. Fig. 1 shows the difference spectrum (difference between optical densities of reduced and oxidized states) of the respiratory pigments in mitochondria of the buccal mass and gizzard muscles of *Aplysia depilans* and *A. limacina*. The absorption bands of cytochromes *a*, *b* and c_1 are clearly shown in the visible region of the spectrum. The trough due to flavoproteins and the Soret bands of cytochromes a_3 and *b* are also clearly visible. Reduction is obtained with dithionite and with more specific reagents like succinate, ascorbate and DPNH. *Aplysia* mitochondria have a strong cytochrome oxidase activity which is 80 % inhibited by carbon monoxide in the dark. The oxygen uptake in presence of succinate is more than doubled when cytochrome *c* is added (4, 5).

Cytochrome *c* was extracted from Aplysia muscles by treatment with acidic ammonium sulfate at 85 % saturation followed by precipitation with TCA, adsorption on Celite, elution and dialysis against running tap water. The pigment

was finally adsorbed on an Amberlite XE-64 column and eluted with ammonium sulfate at 10 % saturation and pH 9.0.

The difference spectrum of the carbon monoxide compound of cytochrome a_3 is given in Fig. 2. Myoglobin which is probably still present in the particles even after repeated washing, interferes with the reaction with CO. Whereas the trough at 444 mμ is very little affected, the peak at 430 mμ of the carbon monoxide compound of cytochrome a_3 is shifted to shorter wavelengths. The suspension was therefore previously treated with ferricyanide, followed by dialysis. The ferric form of myoglobin will not be reduced to ferrous and hence it will not contribute to the CO-spectrum. In this condition the sharp absorption band having its peak at 428 mμ and a trough at 446 mμ clearly define the carbon monoxide compound of cytochrome a_3.

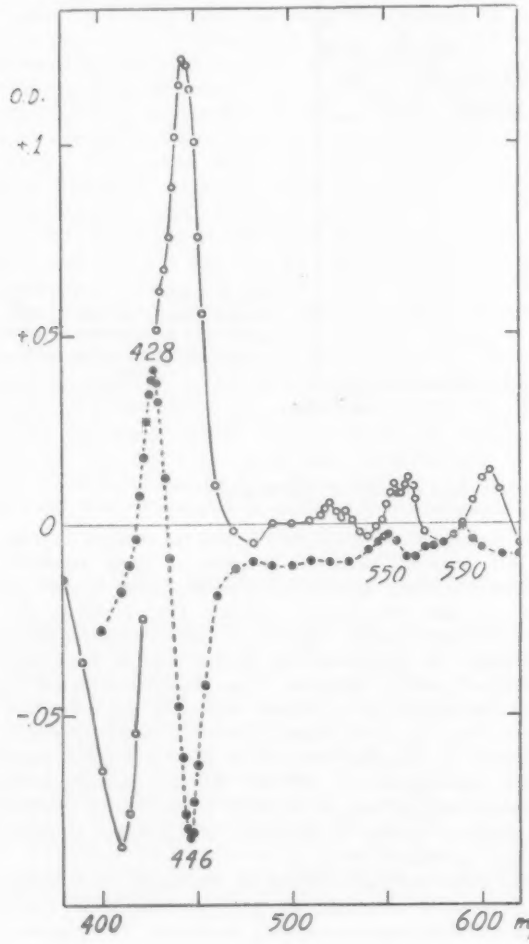

FIG. 2. — *APLYSIA LIMACINA. Buccal muscle particle preparation N° 10. Difference spectra of the carbon monoxide compound after treatment with ferricyanide. Solid line: Succinate treated minus oxidized. Broken line: Succinate plus CO treated minus succinate treated.*

4. The cytochrome h from the hepatopancreas of Aplysia

A pigment can be extracted and purified from the digestive gland of *Aplysia depilans*. It gives a spectrum very similar to cytochrome *h*, both in the reduced and oxidized forms and has several properties in common with the cytochromes of the *b* and *c* groups. Acetone powders of the organ were used. The method of

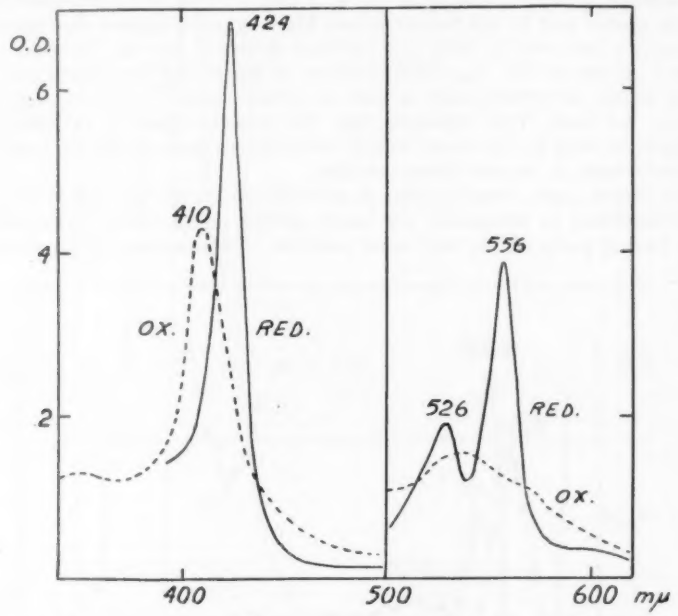

FIG. 3. — *Absorption spectra of Cytochrome* h *from Aplysia hepatopancreas in its oxidized and reduced forms. (Reduction with dithionite).*

preparation was based mainly on extraction with alkaline water, treatment with basic lead acetate and repeated precipitation with ammonium sulfate at 65 % saturation. In its oxidized form, the pigment shows a peak at 410-12 mμ in the Soret region and a broad band with a maximum around 536 mμ in the visible region. Reduction can be obtained with dithionite, lithium hidride, ferrous oxalate, cysteine, glutathione and ascorbate. In the reduced state three bands appear with peaks at 423-25 mμ, 526-28 mμ and 556 mμ as seen in Fig. 3. After treatment with NaOH 0.2 N and pyridine, followed by reduction with dithionite, a spectrum is obtained with peaks at 415, 522 and 553 mμ. Iron porphyrin was extracted only after heating at 80° C. for 30 min and treating with silver sulfate. The spectrum of the acidic porphyrin shows a band with a peak at 395 mμ (6). After reduction with ascorbic acid at pH 7.4 *Aplysia* cytochrome *h* is oxidized by a beef heart cytochrome oxidase preparation in absence of any cytochrome *c*. Added cytochrome *c* increases the rate of oxidation. Beef heart preparation catalyses the reduction of the pigment in the presence of cyanide and of DPNH (6).

5. *The cytochrome system in sea urchin eggs*

The presence of a complete cytochrome system formed by cytochromes *a*, *b*, *c* and a_3 has been demonstrated in mitochondria prepared from unfertilized sea urchin eggs (*Paracentrotus lividus* and *Sphaerechinus granularis*) (7). The difference spectrum obtained after reduction with dithionite or succinate, shows bands with peaks corresponding to the α, β and γ bands of cytochromes *a*, *b* and a_3 in the visible and in the Soret regions. Here again no bands corresponding to cytochrome *c* are visible. However indirect evidence for the presence of cytochrome *c* in sea urchin egg mitochondria is given by the appearance of the reduced bands of cytochromes *a* and a_3 when specific reducing agents, like succinate, are used. This indicates that the concentration of cytochrome *c* in mitochondria, even if very small, is still sufficient to operate the electron transfer from cytochrome *b* to cytochrome oxidase.

The cytochrome system present in unfertilized sea urchin eggs is very similar to that described in mammals, the bands of the components in the difference spectra having peaks at the very same position as those observed for mammalian

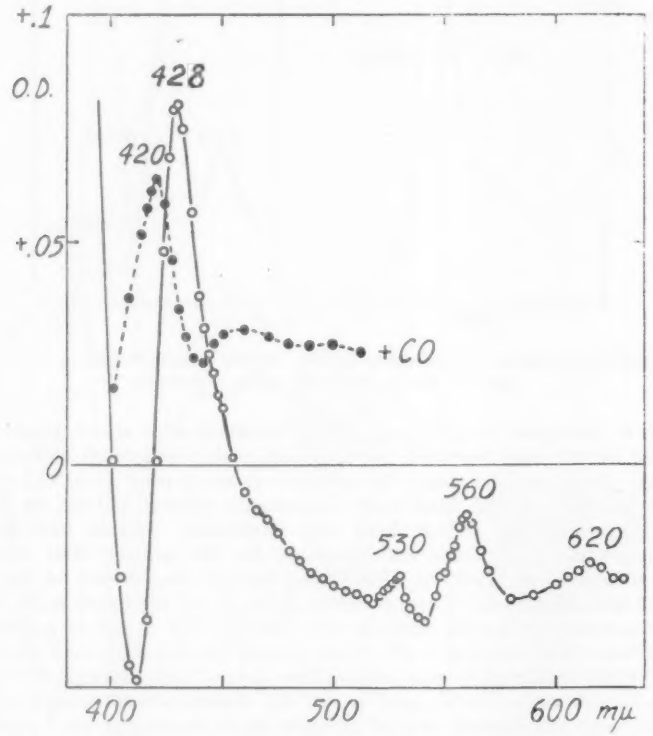

FIG. 1.—*SIPUNCULUS NUDUS. Particles isolated from body muscle. Prep. Nº 4. Solid line: Difference spectrum after reduction with dithionite. Dotted line: The same after treatment with carbon monoxide.*

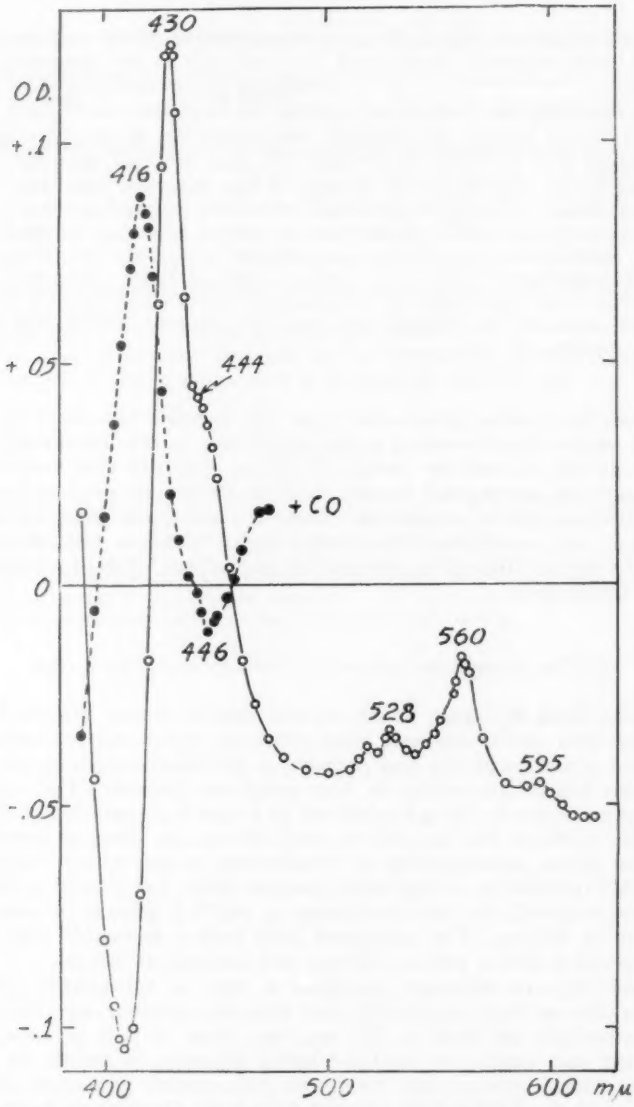

FIG. 5. — *SPIROGRAPHIS SPALLANZANI. Difference spectra of a particle preparation (N° 7) from body muscle. Solid line: Reduction with dithionite. Dotted line: The same after treatment with carbon monoxide.*

TABLE I

Peaks in difference spectra (in mμ) of some groups of marine invertebrates

Invertebrates	Visible					Soret		CO-comp.
	α				β	γ		γ
Octopus vulgaris		605		560	525	444	430	430
Aplysia depilans		605		562 554	527	444	432	428
Paracentrotus lividus		605		562	520	444	430	430
Sipunculus nudus	620			560	530		428	420
Spirographis spallanzani			595	560	528	444	430	416
Heart muscle preparation (*)		605		562	525	444	430	430

(*) This preparation was obtained with the same extraction method used for Marine Invertebrate Muscles.

cytochromes. Sea urchin cytochrome *c* can be replaced by cytochrome *c* from beef heart muscle. Cytochrome a_3 is also very similar to the mammalian pigment. The peak at 430 mμ and the trough at 445 mμ observed after treatment with carbon monoxide, correspond exactly to those known for yeast and mammals.

No difference in the cytochrome system and enzymatic activities was found in fertilized and unfertilized sea urchin eggs. It seems therefore that the cytochromes are not directly responsible for the increase of oxygen uptake which occurs at fertilization.

6. *The cytochrome system of Anellids and Sipunculids*

Particles from the body muscle of two marine worms *Sipunculus nudus* and *Spirographis spallanzani* have been prepared. Sipunculus is known to have hemerithrin, a non porphyrin iron pigment, as the blood oxygen carrier, whereas Spirographis has chlorocruorin, an iron porphyrin pigment. The type of difference spectra shown in Fig. 4 is exhibited by a muscle preparation of *Sipunculus nudus*. The bands at 560 mμ, 530 mμ and 428 mμ are those of cytochrome *b*, whereas no bands corresponding to cytochromes *a* and a_3 are visible. These are probably replaced by a respiratory enzyme which has a band at 620 mμ. It must be remembered that the cytochrome a_2 which is present in some bacteria has a band at 630 mμ. The compound with carbon monoxide gives a rather unusual spectrum with a peak at 420 mμ and a trough at 446 mμ.

Another type of difference spectrum is that of *Spirographis spallanzani* (Fig. 5). In this case there is probably more than one terminal respiratory enzyme: a cytochrome with the band at 595 mμ, very close to that of cytochrome a_1 and another one similar to the CO-binding pigment, as shown by the peak at 416 mμ which appears after treatment with carbon monoxide. Both cytochrome a_1 and the CO-binding pigment have been observed in many types of bacteria. Only the cytochrome oxidase activity was found to give significant values; the DPNH-cytochrome-reductase and the succinic oxidase activities were not detectable by spectrophotometric or manometric methods.

Conclussions and summary

The analysis of the cytochrome system of Marine Invertebrates is still at its very beginning and is therefore very incomplete. However from the results obtained some conclusions can be drawn.

The cytochrome system in the groups examined is the only electron transfer system operating in cell respiration, whatever the oxygen carrier pigment in the blood may be. In some cases the cytochrome system is very similar to that found in yeast and mammals (Molluscs and Echinoderms); in others (worms) it differs greatly and recalls the system described in bacteria (Table I). However the similarity, as indicated by the analysis of the difference spectra, is limited to the system as a whole: the components have in general peculiar properties and characteristics. Only few components have been extracted and purified until now. As indicated by the cytochrome *c* of *Sepia*, they have only few properties in common with the mammalian pigments.

Finally, new respiratory enzymes can be detected in Marine Invertebrates. Cytochrome *h* is a first example and it is probably not the last.

References

(1) Ghiretti-Magaldi, A., Giuditta, A., Ghiretti, F.: *J. cell. comp. Physiol.*, 1958.
(2) Ghiretti-Magaldi, A., Giuditta, A., Ghiretti, F.: *Biochem. J.*, 1957, *66*, 303.
(3) Ghiretti-Magaldi, A., Ghiretti, F.: *Experientia*, 1958, *14*, 170.
(4) Ghiretti, F., Ghiretti-Magaldi, A., Tosi, L.: *J. cell. comp. Physiol.* (In the press).
(5) Tosi, L., Ghiretti-Magaldi, A., Ghiretti, F.: *Rend. Acc. Naz. Lincei*, 1958, *23*, 447.
(6) Tosi, L., Ghiretti, F.: *Experientia*, 1958.
(7) Maggio, R., Ghiretti-Magaldi, A.: *Exp. Cell Res.*, 1958, *15*, 95.

"IN VITRO" SULFATE UPTAKE BY RAT PERITONEAL CELLS

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Our work (7) on the biosynthesis of phenyl sulfates has awakened a great deal of interest in the study of the mechanism of formation of sulfuric acid esters of biological interest. Among these we find the important anticlotting agent heparin, which certainly deserves some special attention because of containing, besides the sulfuric ester bond, the sulfamido bond. A preliminary approach to the problem is that of following the uptake of sulfate by tissues capable of synthesizing heparin. The site of formation of heparin is now generally accepted as being the mast cells, the granules of which give a strong metachromatic reaction (14, 15).

Mast cells are found in many tissues, particularly in subcutaneous tissue and mesenteric preparations As pointed out by Riley and West (22) they are very numerous in the liver capsule of the ox, pig, and dog, that is, in tissues that are almost avascular. According to Padawer (21) the peritoneal fluid of a 200 g. Long-Evans rat contains about 3 ½ per cent mast cells, and that of a Sprague-Dawley rat about 7 to 12 per cent. Rat peritoneal fluid has the advantage of permitting an easy access to mast cells. We have used this type of preparation for the study of sulfate uptake into heparin and presumably chondroitin sulfate.

Uptake of sulfate, probably due to incorporation into heparin, by normal mast cells of the rat skin has been demonstrated by several authors (4, 12, 16, 19) using radioactive sulfate and the radioautographic technique. The uptake by experimental skin tumors has been detected by the same technique by Asboe-Hansen (1) and Boström et al. (3). All the preceding is "in vivo" work, as was that reported by Magnusson and Larsson (20) on the isolation of radioactive heparin from a dog mastocytoma, after intravenous administration of $Na_2S^{35}O_4$ to the dog. Eiber and Danishefsky (9) isolated radioactive heparin from dog's liver after administration of radioactive sulfate to the dog. Sato et al. (24) presented evidence which seems to indicate incorporation of radioactive sulfate into heparin when rat liver slices are incubated with sulfate labeled with S^{35}. The development of a mast cell transplantable tumor in the mouse by Dunn and Potter (8) has permitted Korn (18) and Spolter and Marx (25) to demons-

(*) These studies were aided in party by contract Nonr 1757 (OO), Project No. NR 108-04-8; between the Office of Naval Research, Department of the Navy, U. S. A., and Jefferson Medical College and by research grants A-470 (C4) and A-740 (C5) from the National Institutes of Arthritis and Metabolic Diseases, and grant H-2985 from the National Heart Institute, United States Public Health Service.

trate the "in vitro" incorporation of $S^{35}O_4^=$ into heparin by tumor slices and homogenates. We have used a suspension of cells from the peritoneal fluid of the rat and have been able to demonstrate sulfate uptake into heparin and into what appears to be chondroitin sulfate. The conditions required for this incorporation and the effect of certain inhibitors indicate that the mechanism may be similar to that involved in the formation of the sulfuric acid esters of phenols (7). These results represent the first demonstration of the "in vitro" incorporation of sulfate into heparin by normal mast cells.

Methods

Tissue preparations. The peritoneal cells were obtained from female Sprague-Dawley rats of about 200 grams body weight. The animals were decapitated and then the peritoneal cavity exposed by laparotomy along the mid-ventral line. Four 1 ml portions of a solution of KCl buffered with phosphate (100 ml 0.154 M KCl and 21 ml 0.1 M potassium phosphate buffer pH 7.4) were used to wash the cavity and collect the cells. They were then separated by centrifugation, the supernatant discarded and the cells resuspended in the medium.

The homogenate was prepared from an amount of cells corresponding to 500 000 to 1 000 000 mast cells per vessel, using a Potter-Elvehjem type tissue homogenizer with teflon pestle.

Media. The medium used for intact cells was of the following composition: 95.5 μ moles KCl, 5 μ moles $MgCl_2$, 13 μ moles potassium phosphate buffer pH 7.4, 5.6 μ moles glucose, 200 000 to 1 000 000 counts per second carrier-free $S^{35}O_4^=$ (+) in a volume of 1 ml.

The medium for homogenates had the following components: 95.5 μ moles KCl, 5 μ moles $MgCl_2$, 13 μ moles potassium phosphate buffer pH 7.4, 10 μ moles potassium α-ketoglutarate or succinate, 5 μ moles potassium adenosintriphosphate (**), 100 μg sodium heparinate (***) and 2 000 000 counts per second of carried-free $S^{35}O_4^=$. The addition of sodium heparinate was found necessary and it probably acted as a carrier increasing the recovery of labeled heparin.

Inhibitors or other components of the medium were added at the concentrations indicated below; final volume was 1 ml. Incubation was carried out for 3 ½ hours at 37.5° C.

Preparation of extracts for measurement of radioactivity and paper chromatography. The technique used to extract and purify the heparin and other acid mucopolysaccharides is based on the general principles developed for the extraction of these compounds and follows, at the early stages, the technique developed by Engelberg et al. (10). The incubated material was precipitated with 2 ml methanol plus 2 ml acetone, the precipitate washed twice with 3 ml acetone and processed for digestion as indicated by the above

(+) The $S^{35}O_4^=$ was supplied as sulfuric acid in dilute hydrochloric acid by the Oak Ridge National Laboratory, Oak Ridge, Tenn., U. S. A., on allocation from the U. S. Atomic Energy Commission.

(**) Obtained from Pabst Laboratories, Milwaukee, Wisconsin, U. S. A.

(***) Obtained from Connaught Medical Research Laboratories, Toronto, Canada (1 mg equivalent to 95 to 100 U).

mentioned authors by addition of 20 mg of "Difco" trypsin. After digestion the material was handled according to the technique used by Bassiouni (2) with the following modifications. Ammonium sulfate was added to the extent of 0.5 g per milliliter of solution, the acid polysaccharides were precipitated by addition of a toluidine blue solution instead of an Azure A solution and the precipitate was washed once with 0.01N HCl and twice with distilled water and then extracted twice with formamide. This type of treatment assured a better recovery and also an almost complete elimination of the contaminating radioactive inorganic sulfate. The controls showed values very close to background radioactivity.

Paper chromatography. The technique developed by Kerby (17) using Whatman No. 1 paper and 25 % n-propanol in M/15 phosphate buffer pH 6.4, as a developer for ascending chromatography, was successfully applied. The precipitate obtained with methanol at the end of the extraction procedure, was taken up in 75 μl of triple distilled water, applied to the paper, the chromatogram developed for 17 hours at 4° C., and then dried.

Detection of colored and radioactive spots. The heparin and other acid mucopolysaccharides were located on the dried chromatogram by making use of their metachromatic properties according to the technique of Herman (13) using toluidine blue.

The radioactive spots were detected by scanning the paper strip with an automatic scanner consisting of a Geiger-Müller end-window counter attached to an automatic recorder.

Measurement of radioactivity. The precipitate produced by methanol at the end of the extraction procedure was dissolved in distilled water, and either an aliquot or the total amount was transferred to a stainless steel planchet and evaporated to dryness under an infrared lamp. The counts were determined with a gas-flow chamber attached to an "Atomic" scaler.

Counting of cells. Pooled washings of the peritoneal cavity were centrifuged for 15 minutes at 1 800 r. p. m. to separate the cells. The cell sediment was resuspended in the medium and a sample was taken, mixed with equal volume of Loeffler's methylene blue and placed in a Levy-Hausser counting chamber, with improved Neubauer double ruling, for counting.

Results

Uptake of sulfate by intact cells. For these studies the cells, obtained in the manner indicated above, were suspended in the medium already described and used to an amount corresponding to 500 000 to 1 000 000 mast cells per vessel.

After incubation, the material was processed according to the technique outlined in preceding paragraphs and the extracted acid mucopolysaccharides plated for radioactive counting. These experiments led consistently to a definite high uptake of sulfate, as indicated by the counts of the experimental vessels as compared to the controls. Representative results of a series of experiments are shown in Table I.

Uptake of sulfate by homogenate. Quite irregular results were obtained with the homogenate, some experiments showing positive results and others no incorporation of radioactive sulfate, though the results were consistent for a given preparation. An example of definite uptake is presented in Table II.

TABLE I

Uptake of sulfate by rat peritoneal cells

Medium: 95.5 μ moles KCl; 5 μ moles $MgCl_2$; 13 μ moles potassium phosphate buffer pH 7.4; 5.6 μ moles glucose; 200 000 to 1 000 000 counts per second of carrier-free $S^{35}O_4^=$; 500 000 to 1 000 000 mast cells in a volume of 1 ml. Incubated for 3 ½ hours at 37.5° C.

Vessel	Counts per minute
Experimental	2400 to 5880
Control (not incubated)	30 to 42

As can be seen by comparison with the results on Table I, the homogenate shows about one tenth of the uptake of intact cells.

Paper chromatograms. No radioactivity could be detected in the chromatograms prepared from the material obtained on incubation of homogenates. The preparations derived from incubation of intact cells showed definite colored and radioactive spots. These were compared with simultaneous chromatograms obtained by applying to the paper 5 μg sodium heparinate and 10 μg of chondroitin sulfate (++). Two colored spots were observed, which corresponded to heparin and chondroitin sulfate, with average Rf of 0.2 and 0.9 respectively.

TABLE II

Uptake of sulfate by rat peritoneal cells homogenate

Medium: 95.5 μ moles KCl; 5 μ moles $MgCl_2$; 13 μ moles potassium phosphate buffer pH 7.4; 10 μ moles of potassium α-ketoglutarate or succinate; 5 μ moles of potassium adenosintriphosphate; 100 μg sodium heparinate (equivalent to 0.1 units); 2 000 000 counts per second of carrier-free $S^{35}O_4^=$; homogenate containing 500 000 to 1 000 000 mast cells in a final volume of 1 ml. Incubated for 3 ½ hours at 37.5° C.

Vessel	Counts per minute
Experimental	240 to 570
Control (not incubated)	21.6 to 82.2

Material eluted with distilled water from the spot corresponding to heparin showed anticoagulant activity when tested by the technique of Freeman et al. (11). When the paper strip was scanned for radioactivity two radioactive spots corresponding to the colored spots were found. The peak radioactivity on the

(++) Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio, U. S. A.

(+++) Kindly supplied by the Wallerstein Laboratories, New York, U. S. A.; 0.01 ml of a solution in 75 per cent ethanol added to the vessel and molarity calculated on the basis of a molecular weight of 2 800 (6).

TABLE III

Effect of substrates and glutamine on incorporation of $S^{35}O_4^=$ by rat perotoneal cells

500 000 counts per second of carrier-free $S^{35}O_4^=$; 500 000 to 700 000 mast cells in a volume of 1 ml. with additions as noted.

Preparation	KCl 95.5 μ moles	Mg++ 5 μ moles	K phosphate buffer pH 7.4 13 μ moles	Glucose 5.6 μ moles	D (+) Glucosamine 0.15 μ moles	L-Glutamine 2 μ moles	Incubation (37.5° C) h	Counts per min.
1	+	+	+	+	+	+	0	48
	+	+	+	+	—	—	3 ½	4 720
	+	+	+	+	—	+	3 ½	5 950
2	+	+	+	—	—	—	3 ½	343
	+	+	+	—	+	—	3 ½	799

spot corresponding to an Rf of 0.2 (presumably heparin) varied from 14 to 24 times background and in the other, with an Rf of 0.9, from 20 to 86 times background.

Effect of substrates and glutamine. If the cells were incubated in a medium without any added substrate the uptake was lower than in the presence of either glucose or D (+) glucosamine. The addition of L-glutamine further increased sulfate incorporation. These results are shown in Table III.

Effect of inhibitors. We tested the effect of p-nitrophenol (p-NP), 2,4-dinitrophenol (DNP), methylol gramicidin and molybdate with the results presented in Table IV.

The most pronounced inhibition was obtained with 2,4-dinitrophenol and molybdate; methylol gramicidin (+++) and p-nitrophenol inhibited to a lesser degree.

TABLE IV

Effect of inhibitors on incorporation of $S^{35}O_4^=$ by rat peritoneal cells

Medium: 95.5 μ moles KCl; 5 μ moles $MgCl_2$; 13 μ moles potassium phosphate buffer pH 7.4; 5.6 μ moles glucose; 650 000 to 850 000 mast cells in a volume of 1 ml.

Preparation	Inhibitor	Carrier-free $S^{35}O_4^=$ added counts per second	Incubation (37.5° C) h	Sulfate uptake counts per min.
1	—	1 000 000	0	58
	—	1 000 000	3 ½	8 400
	10^{-2} M Molybdate	1 000 000	3 ½	962
2	—	200 000	0	30
	—	200 000	3 ½	6 600
	2.4×10^{-4} M p-NP	200 000	3 ½	3 950
3	—	500 000	0	46
	—	500 000	3 ½	3 800
	2.5×10^{-4} M DNP	500 000	3 ½	311
	3×10^{-5} M Methylol gramicidin	500 000	3 ½	2 100

Discussion

The results leave no doubt as to the incorporation of sulfate by intact cells. This sulfate appears to be partly incorporated into a compound which seems to be heparin because of its behavior on paper chromatograms and anti-clotting properties. The other chromatographic spot, in which sulfate uptake was detected, appears to correspond to chondroitin sulfate, but additional evidence is needed to confirm this assumption, particularly because of the fact that the chondroitin sulfate used for comparison derives from cartilage. Technical difficulties, and particularly the fragility of mast cells, prevented us from obtaining a direct demonstration of their participation in the process. The results obtained by others (18, 25), with mast cells tumors, strongly indicate that heparin is synthesized by the mast cell. The other substance responsible for sulfate uptake in these experiments, may derive from the activity of the mast cells or of some of the other cells present in the peritoneal cavity of the rat. As observed by Boström and coworkers (5) for costal cartilage, glutamine had a stimulating effect on the sulfate uptake of peritoneal cells. The strong inhibition produced by molybdate is probably due to its interference with the formation of "active sulfate" intermediate (26) and constitutes an indirect evidence for its participation in the incorporation of sulfate by these cells. The uncoupling of phosphorylations is probably responsible for the inhibition by dinitrophenol and methylol gramicidin and this process is known to be required for the introduction of sulfate (7).

p-Nitrophenol may interfere by competing for the "active sulfate" intermediate (3' phosphoadenosine-5'-phosphosulfate) (23).

The considerable decrease or absence of activity in the homogenate is probably the result of dilution and breakdown of required cofactors and substrates needed for the formation of the polysaccharide. We supplied the components known to be needed for the introduction of sulfate (7) but evidently that does not always seem to be sufficient. The inconsistent behavior of the preparation and its low activity makes it difficult to use it for studying biosynthetic processes.

Summary

1) The "in vitro" incorporation of sulfate by rat peritoneal cells has been demonstrated with the use of radioactive sulfate ($S^{35}O_4^{=}$).

2) The homogenate has been found inconsistent in behavior and of low activity when compared to intact cells.

3) The activity of the cells was low in absence of any added substrate and increased on addition of glucose and D (+) glucosamine. L-Glutamine further increased the uptake in presence of glucose.

4) 10^{-2} M Molybdate and 2.5×10^{-4} M dinitrophenol act as strong inhibitors. 3×10^{-6} M Methylol gramicidin and 2.4×10^{-4} M p-NP inhibit to a lesser extent.

References

(1) Asboe-Hansen, G.: *Cancer Res.*, 1954, *14*, 94.
(2) Bassiouni, M.: *J. clin. Path.*, 1954, *7*, 330.
(3) Boström, H., Freiberg, U., Odeblad, E., Ringertz, N.: *Acta path. microbiol. scand.*, 1957, *35*, 1.

(4) Boström, H., Odeblad, E., Friberg, U.: *Acta path. microbiol. scand.*, 1953, *32*, 516.
(5) Boström, H., Rodén, L., Vestermark, A.: *Nature* (Lond.), 1955, *176*, 601.
(6) Cross, R. J., Taggart, J. V., Covo, G. A., Green, D. E.: *J. biol. Chem.*, 1949, *177*, 655.
(7) DeMeio, R. H., Wizerkaniuk, Martha, Fabiani, Emma: *J. biol. Chem.*, 1953, *203*, 257.
(8) Dunn, Thelma, B., Potter, M.: *J. nat. Cancer Inst.*, 1957, *18*, 587.
(9) Eiber, H. B., Danishefsky, I.: *J. biol. Chem.*, 1957, *226*, 721.
(10) Engelberg, H., Dudley, Anne, Freeman, L.: *J. Lab. clin. Med.*, 1955, *46*, 653.
(11) Freeman, L., Engelberg, H., Dudley, Anne: *Amer. J. clin. Path.*, 1954, *24*, 599.
(12) Guidotti, G.: *Exp. Cell Res.*, 1957, *12*, 659.
(13) Herman, D.: *Science*, 1955, *122*, 924.
(14) Holmgren, H., Wilander, O.: *Z. mikr.-anat. Forsch.*, 1937, *42*, 242.
(15) Jorpes, E., Holmgren, H., Wilander, O.: *Z. mikr.-anat. Forsch.*, 1937, *42*, 279.
(16) Jorpes, E., Odeblad, E., Boström, H.: *Acta haemat.*, 1953, *9*, 273.
(17) Kerby, Grace P.: *Proc. Soc. exp. Biol.*, N. Y., 1953, *83*, 263.
(18) Korn, E. D.: *J. Amer. chem. Soc.*, 1958, *80*, 1520.
(19) Lawrence, J. C., Ricketts, C. R.: *Exp. Cell Res.*, 1957, *12*, 633.
(20) Magnusson, S., Larsson, B.: *Acta chem. scand.*, 1955, *9*, 534.
(21) Padawer, J.: *Trans. N. Y. Acad. Sci.* (U. S. A.), Ser. II, 1957, *19*, 690.
(22) Riley, J. F., West, G. B.: *J. Physiol.*, 1952, *119*, 44 P.
(23) Robbins, P. W., Lipmann, F.: *J. biol. Chem.*, 1957, *229*, 837.
(24) Sato, T., Suzuki, T., Fukuyama, T., Yoshikawa, H.: *J. Biochem.*, Tokyo, 1958, *45*, 237.
(25) Spolter, L., Marx, W.: *Fed. Proc.*, 1958, *17*, 314.
(26) Wilson, L. G., Bandurski, R. S.: *J. biol. Chem.*, 1958, *233*, 975.

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milímetro	mm	mm	centímetro cúbico	cm^3	cc
micrón	μ	μ	mililitro	ml	ml
milimicrón	$m\mu$	$m\mu$	kilogramo	kg	kg
Angström	Å	Å	gramo	g	gm
microgramo	μg	μg	miligramo	mg	mg
gama	γ	γ	miliequivalente	mEq	mEq
hora	h	hr	Curie	c	c
minuto	m	Min	Milicurie	mc	mc
	s	sec	Microcurie	μC	μC
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